STUDIES ON THE MECHANISM OF THE PETITE MUTATION IN YEAST

by

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STATEMENT

The results in this thesis are my own, except where due reference is made in the text, and were obtained under the supervision of Dr. G. D. Clark-Walker. The material contained in this thesis has not been presented for the award of any other degree or diploma in any University. Certain sections of the material of this manuscript have already been published (R. M. O'Connor, C. R. McArthur and G. D. Clark-Walker, Eur. J. Biochem. 53 (1975) 137-144; R. M. O'Connor, C. R. McArthur and G. D. Clark-Walker, J. Bact. May (1976). In press). It is intended to submit other sections for publication in the near future.

R. M. O'Connor
This thesis describes the results of research work carried out partly in the Department of Developmental Biology and partly in the Department of Genetics, Research School of Biological Sciences, The Australian National University, during the tenure of an Australian National University Postgraduate Scholarship.
DEDICATION

I dedicate this thesis to my parents and to all members of my family: past, present and future.
ACKNOWLEDGEMENTS

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Finally, but in no way least, I thank Joan Madden for interpreting my writing and producing the excellent quality of the typing of this thesis.
these species, *Torulopsis glabrata*, showed that its mt DNA was 6 μm in length and of buoyant density 1.686 g/cm³. Further, EtBr-induced respiratory-deficient mutants of this species lacked all detectable mt DNA, whilst spontaneous respiratory-deficient mutants were found to have mt DNA of altered buoyant density and smaller circle size. Thus it appears that the spontaneous respiratory-deficient mutants of *T. glabrata* arise by a similar mechanism to petites of *S. cerevisiae*. However, the study emphasises that structural differences between mt DNAs may not be of prime importance in petite-negativity.

Another factor to be considered is the presence of viability genes on the mt DNA of petite-negative yeasts. In this respect, the slow growth phenomenon of petites of *S. cerevisiae* has been studied in order to ascertain if this is associated with the presence of a viability gene on the mt DNA of *S. cerevisiae*. Whilst the results showed that all cytoplasmic petites grew slower than the parent strain in a glucose medium, they also showed that a nuclear respiratory-deficient mutant grew slower than its parent. Further, this slow-growth character is not invariant as repeated sub-culturing of spontaneous petites leads to the isolation of faster-growing variants with growth rates equal to that of the wild-type parent. Thus loss of mitochondrial function in *S. cerevisiae* is slightly inhibitory and in certain circumstances reported in the literature, the loss of mt DNA can be lethal.

In studying suppressiveness, a direct correlation has been found between the spontaneous petite frequency of a strain
and the level of suppressiveness of petites crossed to that strain. Furthermore, the results offer evidence for some form of nuclear dominance operating in suppressive crosses. This relationship between petite frequency and level of suppressiveness is discussed in terms of the enzymes responsible for the excision-insertion processes and the structure of the mt DNA.

It appears that petite-negativity may be due to a number of factors, such as mt DNA structure, presence of viability genes or lack of enzymes catalysing the excision-insertion processes. It also seems that more than one factor operates in determining the suppressiveness of a petite mutant.
ABBREVIATIONS

CAP chloramphenicol
CsCl caesium chloride
DNA deoxyribonucleic acid
mt DNA mitochondrial deoxyribonucleic acid
o DNA omicron deoxyribonucleic acid
ERY erythromycin
EtBr ethidium bromide
\( \mu m \) micrometre (10^{-6} \text{ metre})
R.D. respiratory-deficient
RNA ribonucleic acid
s[rho]^{-} spontaneously-arisen petite mutant
e[rho]^{-} ethidium-bromide induced petite mutant
f[rho]^{-} faster-growing variant of a spontaneously-arisen petite mutant
SSC standard saline citrate
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CHAPTER 0

INTRODUCTION
SECTION A - GENERAL INTRODUCTION

(A.1) Early studies

The first observation of yeast cells using a light microscope was made by van Leeuwenhoek who reported seeing "animalcules" in a drop of fermenting beer. In 1870, Reess proposed the name *Saccharomyces* (Greek *Sakchari*, sugar + *Mukes*, a mushroom) for the spore-forming yeasts and included them in the ascomycetes. Yeasts of the genus *Saccharomyces* are probably the most commonly known of all yeasts, being used extensively in the production of beer, wine and bread and include the top-fermenting yeast *S. cerevisiae* and the bottom-fermenting yeast *S. carlsbergensis*.

Pasteur studied the fermentation process of fungi and in his "Etudes sur la bière" in 1876, postulated that the fermentation process for organisms living under anaerobic conditions constituted a substitute for the respiratory process of aerobic organisms. Pasteur summarized his observations in his well-known phrase: "La fermentation est la vie sans air".

(A.2) Fermentation in fungi

Fungi flourish in the presence of air, but if deprived, some can gain enough energy by fermentation for the maintenance of life. However, only two major groups, yeasts and mucors, can gain enough energy from fermentation for active growth and division. Of these two groups, some yeasts have evolved the property of glucose repression (Ephrussi *et al.* 1956; Gordon and Stewart 1969; Kaplan and Criddle 1970) which is not present in mucors (Clark-
Walker 1973A). This property enables some yeasts to have repressed mitochondria in very dilute sugar solutions (0.1% glucose), whereupon the organism grows solely by fermentation even in the presence of air. Yeast cultures possessing this property are termed "facultatively-fermentative" whilst respiratory-deficient yeast mutants, discussed below, are "obligately-fermentative". These respiratory-deficient mutants depend solely on fermentation for growth and division.

This ability to grow in the absence of air, provided that the medium is supplemented with ergosterol and unsaturated fatty acids (Andreasen and Steir 1953, 1954) is one of the unusual properties of \textit{S. cerevisiae} and allows for the occurrence of a state of respiratory deficiency. This feature is the underlying basis for the observation of non-lethal mutants having this property.

SECTIONS B – THE PETITE MUTATION

The petite mutation is a cytoplasmically-inherited highly pleiotropic, non-reverting mutation, resulting in respiratory deficiency and the loss of the \([\rho]\) factor \((\rho)\) (c.f. below), which occurs spontaneously at a high rate (around 1%) in certain strains of yeast (Ephrussi 1953).

\section{Early work}

Although several American researchers had previously reported the occurrence of respiratory deficiencies in yeast, a non-Mendelian inherited mutation resulting in the loss of the capacity for respiration in \textit{S. cerevisiae} was reported by Ephrussi \textit{et al.} (1949A). Later the French investigators designated the small, white colonies that
arose as a result of the mutation, as "petite" (small) and the large, cream-coloured, wild-type parent "grande" (large) (Ephrussi 1953) (photo 0-1). The early literature is documented in an extensive review (Nagai et al. 1961).

(a) Non-Mendelian character

The first demonstration that the petite mutation was inherited in a non-Mendelian manner, came from a series of papers by Ephrussi and co-workers (Ephrussi and Hottinguer 1950; Ephrussi et al. 1949A, B, C; Slonimski and Ephrussi 1949). This series of papers demonstrated that when petite cells of either mating type were crossed with wild-type cells, diploids were produced which were respiratory-competent, like the wild-type parent. On sporulation of the diploid cells, all four ascospores were found to be respiratory-competent. Wild-type cells of the F₁ generation were chosen at random and backcrossed to the original petite parent; all the progeny were respiratory-competent. The same procedure was carried out for four backcross generations and the petite phenotype appeared in only 5 out of 596 ascospores. The few mutants arising were shown by a statistical treatment, to be probably new mutants. The authors concluded that the genetic analysis is compatible with a non-Mendelian mode of inheritance for the petite mutation.

The convincing demonstration of the cytoplasmic nature of the petite determinant was achieved by use of a heterokaryon system (Wright and Lederberg 1956). The authors took advantage of a transient heterokaryon system that occurs during zygote formation in some S. cerevisiae strains. In this case, when the haploid strains are
PHOTO 0-1

The *petite* phenotype

Colonies of *S. cerevisiae* wild-type strain 4342 grown in ETYP and plated onto selective medium GGYP. Note the different morphology apparent between the large, cream-coloured *grande* colonies and the smaller, whiter *petite* colonies. Strain 4342 has a spontaneous *petite* frequency of 1%. 
crossed, there is a period during cell fusion in which buds are produced before the nuclei have fused. Thus, occasionally buds arise containing one of the unfused parental nuclei in a mixed cytoplasm. Such buds then form haploid cell lines with a nucleus from one parent and a cytoplasm from both. Using strains with different auxotrophic mutations, Wright and Lederberg were able to transfer the \textit{petite} character from one strain to the other in 6 out of 530 pairs and vice-versa, transferring the wild-type character in 5 out of 530 pairs. Thus it was shown that the \textit{petite} phenotype segregates independently of the nucleus.

(b) Physiological aspects

The description of the basic \textit{petite} phenotype was carried out principally by Slonimski, working with a \textit{petite} strain used in Ephrussi's genetical analysis (Ephrussi 1952; Ephrussi and Slonimski 1950; Slonimski 1949A, B; Slonimski and Ephrussi 1949). \textit{Petite} mutants were identified initially by their inability to grow on a non-fermentable carbon source and by the smaller colonies which they formed on glucose plates. Under aerobic conditions and in the presence of glucose, wild-type yeast cells grew faster than did the \textit{petite} cells and produced about four times as much cell mass.

The absence of respiratory activity in the \textit{petite} mutants was shown to result from an absence of the electron transport chain. Spectral studies showed that \textit{petite} mutants had neither cytochrome b nor cytochrome a + a\textsubscript{3}, while the level of cytochrome c was higher than in respiratory-competent cells. Cytochrome c, and a number of the dehydrogenases and enzymes of the Krebs cycle which are
soluble mitochondrial enzymes, are present in both wild-type and petite strains (Clark-Walker and Linnane 1967, Claisse and Pajot 1974).

Later work showed that all petite cells have mitochondria which lack a functional mitochondrial protein synthesising system (Schatz and Saltgaber 1969) but which in most cases still retain the ability to transcribe the mitochondrial DNA (mt DNA) (Lamb and Rojanapo 1973, Cohen et al. 1972). The mitochondrial protein synthesising system has been shown to be blocked at the level of translation in petite cells (Kuzela and Fecikova 1970), but only some petites lack functional ribosomes (Wintersberger and Viehauser 1968). This lack of functional ribosomes would explain the loss of certain mitochondrially coded proteins in some petite cells but it doesn't explain why the other petite cells and some nuclear respiratory-deficient mutants show the same pleiotropic effects, the most important being the lower growth rate as discussed below.

(B.2) Detection and induction of petite mutants

The simplest technique employed to detect petite colonies involves the use of a selective growth medium, GGYP (Appendix I) which depends on the inability of petite colonies to grow on a non-fermentable substrate. This medium, of 4% glycerol and 0.2% glucose, provides enough glucose for the respiratory-deficient mutants to form small, white, colonies and allows the wild-type, respiratory-competent cells to form large, cream-coloured colonies (photo 0-1). A second common method of detection uses an overlayer technique with molten agar containing 0.05% 2,3,5 triphenyl-tetrazolium chloride (TTC). The wild-type
colonies develop a deep-red colouration due to the reduction of the TTC to a formazan by a functional electron transport chain whilst the petite colonies, which lack an electron transport chain, remain colourless (Ogur et al. 1957).

Studies on the induction of the petite mutation suggested that a DNA species of the yeast cell was the site of action of the petite mutation. Monochromatic ultraviolet radiation induces the petite phenotype at a very high rate, with a maximum effect around 260 nm implicating a nucleic acid as target, whilst X-rays cause very little increase in the petite frequency, (Raut and Simpson 1955). To date, the most efficient inducers of the petite genotype are planar, usually basic, polycyclic aromatic molecules such as substituted acridines, e.g. euflavine (Ephrussi 1953); anthracenes, e.g. dithranol (Gillberg et al. 1967) or phenathridines, e.g. ethidium bromide (Slonimski et al. 1968). Such molecules are known to be capable of strong interactions with DNA leading to profound distortions of the macromolecule (Crawford and Waring 1967, Le Pecq and Paoletti 1967), by intercalation between the stacked bases on the double helix (Krugh and Reinhardt 1975).

Elevated temperature also effectively induces the petite mutation. Ycas (1956) reported that growth at 40°C led to a culture of nearly 100% petite colonies and Sherman (1959) confirmed this and also showed that heat shock, growing the cells at 30°C and then shifting the culture to 54°C, was equally effective. A vast array of unrelated compounds, including the dye TTC (Laskowski 1954)
have also been reported as inducing the petite mutation

(B.3) Segregational respiratory-deficient mutants

The term rho factor (ρ) was introduced to distinguish the more commonly occurring cytoplasmic respiratory-deficient mutants from the rarer chromosomally-inherited, respiratory-deficient mutants (segregational petites) (Chen et al. 1950, Sherman 1963). Sherman called the cytoplasmic determinant for respiratory competence in yeast the rho factor (ρ) and the corresponding nuclear determinant the P factor (P). A recent suggestion indicates that ρ should be replaced by [rho] and P by pet− (Pliscke et al. 1975). Thus wild-type, respiratory-competent strains of yeast are annotated pet+ [rho]+, segregational-petite strains are pet− [rho]+, cytoplasmic petite strains are pet+ [rho]− and double mutants are pet− [rho]−.

The occurrence of nuclear gene mutations which confer the respiratory-deficient phenotype in S. cerevisiae was first reported by Chen et al. (1950). To date, 25 separate complementation groups, designated pet1 to pet46, have been described in the nuclear genome which give rise to segregational-petite strains (Lachowicz et al. 1969; Kotylak 1973; Pliscke et al. 1975). However, for the purposes of this thesis, the term petite will refer only to the cytoplasmic mutation.
Suppressiveness

Suppressiveness is a measure of the ability of cytoplasmic petites to suppress the transmission of the respiratory sufficient character of an opposite mating-type strain to the diploids resulting from a cross of the two strains. Petite strains are now known to vary from 0% suppressive (neutral petites) to greater than 99% suppressive (highly suppressive petites). The early studies of Ephrussi and co-workers (Ephrussi 1953) used acriflavine-induced petites, now known to be neutral petites, and hence the phenomenon of suppressiveness was not reported until some time later. The term suppressiveness was then introduced on the basis of a model in which the wild-type grande cell contained a cytoplasmic factor which was lacking in the neutral petite strains. In the case of suppressive petite strains it was conceived that the cytoplasmic factor of the grande cell was supplanted or suppressed by a factor derived from the suppressive petite cell (Ephrussi et al. 1955).

Subsequent work has revealed that the phenomenon of suppressiveness is complicated not only by the range of the degree of suppressiveness found, but also by the phenomenon of 'delayed expression' in the diploids formed in such crosses. Thus if a neutral petite strain is crossed with a grande strain, the resulting diploids are all grande and yield only respiratory competent ascospores upon sporulation (tetrad ratios of 4:0; wild-type:petite). On the other hand, if a suppressive petite strain is crossed with a grande strain, the suppression of respiratory competence is not immediate; if sporulation is induced shortly after crossing...
two types of asci can be obtained. In one instance the
asci will contain only petite ascospores (tetrad ratios of
0:4; wild-type to petite) whereas in the other instance
the asci will contain only grande ascospores. It was
further reported that if sporulation was delayed, then it
is found that asci contain only [rho]+ ascospores due to
the apparent inability of [rho]− diploids to sporulate
(Ephrussi and Grandchamp 1965). Later work has shown that
sporulation can occur in the absence of a functional mito-
chondrial genetic system provided the cells are fully
respiratory active prior to transfer to sporulation medium.
Hence the mitochondrial genome does not contain genes
directly required for sporulation (Kuenzi et al. 1974).

Furthermore, a series of petite strains generated
by repeated subcloning of an established petite strain,
showed a strong mother-daughter correlation in degree of
suppressiveness when all strains were crossed to the same
opposite mating-type [rho]+ strain (Ephrussi and Grandchamp
1965) and a later study showed that the diploid cells, which
are initially heterozygous for the petite and wild-type
phenotype, produce segregants that are pure [rho]+ or pure
[rho]− during clonal growth to form colonies (Ephrussi et
al. 1966).

As yet, the molecular mechanism of suppressiveness
is unknown, but a recent hypothesis has been advanced (Clark-
Walker and Miklos 1974B) as discussed below.
SECTION C - MITOCHONDRIAL DNA (mt DNA) IN S. CEREVISIAE

(C.1) mt DNA in other organisms and fungi

Identification of DNA in mitochondria was not reported until the early 1960's (Nass and Nass 1963A, B; Luck and Reich 1964). Since that time, mitochondrial DNAs from many different organisms have been studied and, in the case of the higher animal systems, shown to be strikingly similar in size, topology and mode of replication. In higher animals the mt DNAs have all been found to be circular, varying slightly in size, but having a molecular weight of around $10^7$ daltons or 5 μm (Borst and Flavell 1972A; Borst 1972; Katsamatsu and Vinograd 1974). These characteristics of mitochondrial DNA occur only in higher animals and do not extend to plants and protists. For example, mt DNA size can range from the small circles, 0.3 μm, seen in the kinoplast of the flagellate protozoa, Leishmania tarentolae (Wesley and Simpson 1973A, B, C) to 13 μm circles in the protozoan Acanthamoeba castellani (Bohnert 1973; Bohnert and Herrmann 1974). Further, the mt DNA of the ciliate protozoan, Tetrahymena pyriformis exists as a 15 μm linear duplex rather than as a circular form (Suyama and Miura 1968, Flavell and Follett 1970). In the only higher plant studied, mitochondria from pea leaves have a large circular DNA of 30 μm (Kolodner and Tewari 1972).

Buoyant density studies on mt DNA have shown that no mt DNA with a G + C content above 50% is known, although G + C contents below 30% are quite common and in some yeast petite strains can go below 5%. There is no obvious correlation between the base composition of mt DNA and nuclear DNA; the base composition of mt DNA may be higher,
the same or lower than that of nuclear DNA (Borst 1972).

Further to the situation existing with the mt DNA of higher animals, the mt DNA of diverse species of fungi has been shown to occur in the form of covalently-closed circular DNA varying in size from 14 to 25 μm (Hollenberg et al. 1970; Clayton and Brambl 1972; Agsteribbe et al. 1972; Clark-Walker and Gleason 1973). In wild-type strains of yeast, the mt DNA is circular and 21-25 μm in length, (Hollenberg et al. 1970; Petes et al. 1973), whilst in some petites the mt DNA is also circular but of a much smaller size (Bernardi et al. 1968; Hollenberg et al. 1972A; Clark-Walker and Miklos 1974A; Locker et al. 1974).

(C.2) **Unique properties of *S. cerevisiae* mt DNA**

The unique properties of *S. cerevisiae* mt DNA centre around both its large size (length 21-25 μm) (Petes et al. 1973; Hollenberg et al. 1970) and also its relatively high A+T content. The A+T content of *S. cerevisiae* mt DNA has been found to vary depending on the method of determination, being 82% by chemical analysis, 87% by melting-point determination, and 76% by buoyant density analysis (Bernardi et al. 1970), in comparison to the known upper limit of 75% A+T for bacterial DNA, implicating the presence of non-coding sequences in the mt DNA of *S. cerevisiae*.

Extensive investigations into the structure of yeast mt DNA by Bernardi and co-workers has shown that *grande* mt DNA has AT-rich sequences interspersed with relatively GC-rich sequences. The melting-profile of *grande* mt DNA shows a compositional heterogeneity, suggesting that AT-rich sequences could comprise up to 50% of the genome (Bernardi et al. 1968, 1972), whilst buoyant density studies show the same compositional heterogeneity with four separate peaks being
resolved from sonicated wild-type mt DNA in CsCl gradients and four again from unsonicated wild-type mt DNA in Ag⁺-(Cs)₂SO₄ gradients (Carnevali and Leoni 1972; Vedel et al. 1972). The observed compositional heterogeneity was postulated to be due to AT-rich sequences interspersed in the grande mt DNA (Piperno et al. 1972).

Using both spleen acid DNAase, which splits mt DNA with a slight preference for the G + C-rich regions, and micrococcal nuclease, which splits mt DNA with a very high specificity for the A + T-rich regions, Prunell and Bernardi (1974) have isolated both AT-rich sequences, homogeneous in base composition, with a G + C content lower than 5% and a minimum length of 1.6 x 10⁵ daltons, and also G + C-rich sequences heterogeneous in base composition, with a G + C content ranging from 25-50% and an average size of 1.2 x 10⁵ daltons. Previously it was shown that sheared yeast mt DNA has unimodal, symmetrical bands of unaltered buoyant density in a CsCl gradient at molecular weights as low as 2 x 10⁶ daltons (Bernardi et al. 1972), whilst Locker et al. (1974) have shown that the mt DNA of petite strains must have a circle size less than 2 μm before there is any detectable change in the buoyant density of the mt DNA. Hence it would appear that the lengths of these "spacer" regions lie in the range 1.6 x 10⁵-2 x 10⁶ daltons.

(C.3) The petite mutation and mt DNA

(a) Buoyant density studies

DNA isolated from a whole homogenate of yeast cells was shown to have a light satellite DNA peak of buoyant density 1.684 g/cm³ which was demonstrated to be of mitochondrial origin (Moustacchi and Williamson 1966; Tewari et al. 1966). A correlation between the petite phenotype and
mt DNA came from a study of the mt DNA of a series of established petite strains which were shown to have mt DNA of altered buoyant density (Mounolou et al. 1966). In this study most petites had mt DNA of a lighter buoyant density (higher mole percent A + T) but in one case there was a shift to a higher value (higher G + C). Although more reports of shifts to a higher buoyant density are known for petites, in general the shift is to a lighter buoyant density (Locker et al. 1974).

Further association between the petite mutation and mt DNA was shown by using the intercalative dye ethidium bromide (EtBr). Petite strains generated by EtBr treatment do not contain detectable mt DNA, furthermore these strains have zero suppressiveness (neutral petites) (Goldring et al. 1970; Nagley and Linnane 1970). Subsequent studies showed that long periods of exposure to EtBr leads to cultures composed entirely of neutral petite colonies, whilst lower concentrations of the mutagen or less exposure time, results in isolation of petite colonies retaining mt DNA which has varying buoyant density (Goldring et al. 1971). These studies firmly equate the rho factor with mt DNA and draw an association between the petite mutation and the loss or alteration in the composition of mt DNA.

Extensive studies on the mt DNA of a low buoyant density, ethidium-bromide induced petite mutant RD_{1A} has shown that the mt DNA (G + C = 3 mole percent) consists of a perfect repeating-unit, about 70 nucleotides long, amplified many times to form both long linear DNA and also large fishnet concatenanes. Quantitative renaturation studies showed that the RD_{1A} mt DNA renatures about 300 times faster than wild-type mt DNA and is complementary to about
0.5% of the wild-type mt DNA, suggesting that RD<sub>1A</sub> mt DNA represents the amplification of one small segment of the wild-type mitochondrial genome (Hollenberg <i>et al.</i> 1972B, Mol <i>et al.</i> 1974).

Thus it would appear that not only does the wild-type mt DNA of <i>S. cerevisiae</i> contain AT-rich sequences interspersed in its genome, but that the mt DNA of low buoyant density <i>petite</i> strains consists of a small segment of this genome amplified many times. This latter point is demonstrated more clearly by hybridization and renaturation studies.

(b) Hybridization and renaturation studies

The use of hybridization and renaturation techniques have provided the strongest evidence so far for sequence loss and re-arrangement in the mitochondrial genome of <i>petite</i> strains.

The mt DNA of yeast wild-type strains has been shown to code for mitochondrial r-RNA, t-RNA and a number of hydrophobic proteins that form part of the inner mitochondrial membrane (Borst and Flavell 1972B; Rabinowitz and Swift 1970; Cohen and Rabinowitz 1972; Casey <i>et al.</i> 1972). These mitochondrial r-RNAs and t-RNAs differ in both physical and hybridization properties to their counterparts in the cytoplasm, with the mitochondrial RNAs hybridizing specifically to the mt DNA (Borst and Grivell 1971, Borst 1972). Hybridization of the mt RNA with wild-type mt DNA indicates the presence of coding capacity for 14 genes for t-RNA on the yeast mt DNA, compared with 11 such cistrons on <i>HeLa</i> mt DNA, 15 on <i>Xenopus</i> mt DNA and the 33 t-RNAs minimally necessary to read the 61 possible codons with maximum wobble
Ten yeast t-RNAs have been identified as hybridizing with the wild-type mt DNA, these being: methionine -, f-methionine -, leucine -, valine -, alanine -, phenylalanine -, isoleucine -, glycine -, serine - and tryptophan-t-RNA (Halbreich and Rabinowitz 1971; Cohen and Rabinowitz 1972).

An analysis of a series of spontaneous petite strains derived from the same wild-type strain showed that in all cases the mt DNA retained the cistron for leucine-t-RNA and all but two petites retained the cistron for alanine-t-RNA. Only one petite strain, whose mt DNA was of less altered buoyant density retained the cistrons for valine - and isoleucine-t-RNA (Cohen et al. 1972). In another study, the mt DNA of a low-density petite strain (buoyant density of the mt DNA was 1.671 g/cm³) was shown to retain the cistron coding for serine-t-RNA but had lost the cistron for phenylalanine-t-RNA (Carnevali et al. 1973).

Renaturation kinetics cannot show the same codon-specific deletions in spontaneous petite strains, but can still indicate loss of sequences in petite mt DNA that is not detectable by buoyant density analysis (Fauman and Rabinowitz 1972). The mt DNA from EtBr-induced petite strains had renaturation rates 2-600 times faster than that of the grande mt DNA, indicating a gross reduction in the complexity of the mt DNA, suggesting considerable deletions.

On the other hand, the presence of non-homologous or petite-unique sequences on the mt DNA of some petite strains has been implied both from mt RNA:mt DNA hybridization studies (Fukuhara et al. 1969, Fauman et al. 1973) and from mt DNA:mt DNA hybridization studies (Gordon and
Rabinowitz 1973). However, it is feasible that these petite-unique sequences arise by some unknown re-arrangement of the mt DNA sequences present in the grande mt DNA.

(c) Suppressiveness studies

As previously introduced, another characteristic that has been used to differentiate various cytoplasmic petites is that of suppressiveness, which is measured as the percentage of entirely petite zygotic colonies obtained in a cross of the petite with a [rho]+ haploid. A number of authors studied the relationship of the degree of suppressiveness to the extent of the changes observed in the buoyant density of the mt DNA (Mounolou et al. 1966; Mehrota and Mahler 1968; Carnevali et al. 1969). Although the results were equivocal, the large alterations in some highly suppressive petites led Carnevali et al. (1969), to suggest that the more grossly altered the mt DNA, the higher would be the suppressiveness of the petite. A later study supported strongly the assumption that the phenomenon of suppressiveness is associated with the presence of mt DNA. However, no correlation could be found between the buoyant density of the mt DNA and the degree of suppressiveness of the petite (Michaelis et al. 1971). Two further studies also failed to find any correlation between the degree of suppressiveness of the petite strains analysed and the buoyant density, size or proportion of their mt DNA (Michels et al. 1974, Tingle et al. 1974). Furthermore, in a cross of a suppressive petite having mt DNA of altered buoyant density, by a normal respiratory competent strain, the resulting diploids can have mt DNA of intermediate buoyant density (Carnevali et al. 1969, Shannon et al. 1972).
In conclusion, it has been demonstrated that neutral petites lack all detectable mt DNA and that the phenomenon of suppressiveness is associated with the presence of mt DNA. However, no correlation seems to exist between the buoyant density of the mt DNA and the degree of suppressiveness of a petite strain.

SECTION D - CYTOPLASMIC GENES IN S. CEREVISIAE

(D.1) Occurrence of cytoplasmic genes in other organisms

Cytoplasmic genes were recognised at the turn of the century, with independent reports from Correns (1909) and Baur (1909), each describing the non-Mendelian inheritance of a factor influencing chloroplast development; in both cases the phenotypes involved leaf variegation. Later studies concentrated on higher plants and produced evidence of non-Mendelian genes influencing chloroplast development, pollen sterility and other morphogenetic properties. Only recently has the study of cytoplasmic genes turned to simple organisms with a quick, sexual life cycle, and the cytoplasmic systems most investigated are those in the yeast S. cerevisiae and the green alga Chlamydomonas (Sager 1972).

Initially, cytoplasmic genes were identified by their failure to follow Mendelian laws of inheritance, and at present the principal criteria for identification of cytoplasmic genes are: (a) non-Mendelian inheritance, (b) independence of nuclear and cytoplasmic gene assortment in suitable systems, e.g. heterokaryons, (c) differences in the progeny of reciprocal crosses, (d) extensive somatic segregation during vegetative growth (Sager 1972).
(D.2) Types of cytoplasmic genes in *S. cerevisiae*

(a) Mitochondrial genes and recombination

The discovery that antibiotics such as chloramphenicol and erythromycin, which inhibit protein synthesis in bacteria, prevent synthesis of respiratory enzymes in yeast (Clark-Walker and Linnane 1966, Huang *et al.* 1966) initiated studies on mitochondrial genes and recombination in *S. cerevisiae*. In the presence of the antibiotic, the wild-type sensitive strains can grow on glucose but not on non-fermentable substrates. It is then possible to select for resistant mutants on a medium containing glycerol, together with the drug (Wilkie *et al.* 1967). The resistant colonies selected for on the antibiotic-containing plates, arise from clones already present in the culture and are not induced by the antibiotic (Birky 1973).

In *S. cerevisiae* two classes of mitochondrial-encoded drug-resistant mutants have been described, firstly, those mutants resistant to inhibitors of mitochondrial protein synthesis, e.g. chloramphenicol, erythromycin, spiramycin (Linnane *et al.* 1968B) and secondly, those mutants resistant to inhibitors or uncouplers of oxidative phosphorylation (Griffiths *et al.* 1974).

To date, there are 15 known mitochondrial genes in *S. cerevisiae*, of which twelve confer resistance to drugs, one is the [rho] factor itself, one is a temperature sensitive mutation [TSM8] associated with an enhanced mutation rate to [rho]− (Handwerker *et al.* 1973) and the last is [ome], which specifies "mitochondrial sex" (Coen *et al.* 1970; Bolotin *et al.* 1971). Of these 15 known genes, 10 genes can be mapped on 3 separate linkage groups, whilst
the five remaining genes have not been ascribed to any linkage group (Plischke et al. 1975).

The first linkage group contains the genes [RIB1] (chloramphenicol resistance), [RIB2] (erythromycin and/or spiramycin resistance), [RIB3] (erythromycin and/or spiramycin and/or chloramphenicol resistance) all of which specify the mitochondrial ribosome, plus the temperature-sensitive gene [TSM8] and the "mitochondrial sex" gene [ome].

The second linkage group contains the genes [OLI1] (resistant to oligomycin) which specifies the mitochondrial membrane ATPase complex, [OLI3] (resistant to oligomycin) and [VEN1] (resistant to venturicidin).

The third linkage group contains the two genes [OLI2] and [OLI4], both of which confer oligomycin resistance.

Five other genes, not located on any linkage group are [EBR1] (resistance to ethidium bromide), [MIK1] (resistant to mikomycin), [PAR I] (resistance to paromomycin), [TCR1] (resistant to tetracycline) and the [rho] factor itself.

The first demonstration of recombination between mitochondrial genetic markers used strains resistant to spiramycin, paromomycin and erythromycin and the results of these crosses demonstrated the recovery of recombinant genotypes (Thomas and Wilkie 1968). Later studies demonstrated by sporulation tests on the recombinant progeny that recombination had occurred at the mt DNA level (Coen et al. 1970; Rank 1973; Bolotin et al. 1971).

The study of recombination in mitochondrial genes
is complicated by the features of polarity of recombination and polarity of transmission. Polarity is defined as a deviation from the expected 1:1 recovery of alleles in reciprocal crosses. The basis of recombinational polarity has been attributed to a pair of genetic factors, the omega factor \([\text{ome}]\), determining mitochondrial sexuality, which segregate independently of the chromosomally-coded mating-type alleles. This polarity is only seen in heterosexual crosses \([\text{ome}]^+ \times [\text{ome}]^-\) and not in homosexual crosses \([\text{ome}]^+ \times [\text{ome}]^+\) or \([\text{ome}]^- \times [\text{ome}]^-\). In heterosexual crosses, the distribution of markers at the \(\text{CAP}^R\) and \(\text{ERY}^R\) loci are always skewed in favour of the marker linked in the parents to the \([\text{ome}]^+\) allele (Coen et al. 1970, Bolotin et al. 1971). \([\text{Ome}]\) has been mapped on linkage group 1 along with \([\text{RIB1}]\) and \([\text{RIB2}]\), but controversy exists at present over its exact location with respect to these two loci (Howell et al. 1974, Deutsch et al. 1974). An alternative hypothesis to explain polarity of recombination has been advanced, invoking intra-molecular rearrangements of the mitochondrial genome (Clark-Walker and Miklos 1974B).

The second aspect, polarity of transmission (bias) is the preferential transmission of mitochondrial genes from one parent and is most clearly seen in homosexual crosses (in which both parents carry the same allele at the \([\text{ome}]\) locus) (Avner et al. 1973). This preferential recovery of mitochondrial genomes from a particular parental strain is under the control of a series of nuclear genes which influence the segregation processes undergone by the mitochondrial genomes in both homosexual and hetero-
sexual crosses, without modifying the main features controlled by the mitochondrial [ome]$^+$/[ome]$^-$ system (Avner et al. 1973). A recent report indicates that both bias and polarity of recombination can be modified by glucose repression (Birky 1975).

(b) Other extrachromosomal genes

In addition to the known mitochondrial genes described above, there exist four cytoplasmic genes in *S. cerevisiae* that have been shown to be non-mitochondrial. These genes are the killer factor, designated [k] and [n] (Bevan and Makower 1963), a cytoplasmic suppressor, designated [psi] (Cox 1965), a triethyl tin resistant gene, designated [TET 1] (Lancashire and Griffiths 1971) and a gene conferring the ability to incorporate ureidosuccinic acid, designated [URE 3] (Lacroute 1971).

In the first example, that of killer, the presence or absence of this factor leads to three different yeast cell types: killers, sensitive and neutrals. Sensitive cells are killed when mixed and grown together with killer cells. The neutral cells are resistant to the toxic agent of the killer cells, and in turn show no toxicity towards the sensitive cells (Bevan and Makower 1963). The toxic agent has been characterized as a protein.

The killer phenotype is determined by a cytoplasmic factor [K] and a nuclear gene M controlling the maintenance of [K]. Another cytoplasmic factor [n] confers the neutral phenotype and again M is needed for its maintenance. Cells without a cytoplasmic factor are sensitive, they can be either M or m with respect to the nuclear gene. The search for the genetic determinant of the cytoplasmically-inherited
killer factor, led to the isolation of two high molecular weight species of double-stranded RNA (dsRNA) which are associated with isometric virus-like particles and have been shown by genetic evidence, to determine the killer factor (Bevan et al. 1973; Vodkin et al. 1974; Herring and Bevan 1974).

The [psi] gene was described in terms of a stable cytoplasmic mutation which interferes with the action of nuclear super-suppressors (Cox 1965). [psi]+ is necessary for the expression of the nuclear super-suppressor SUQ5. Super-suppression in yeast is probably caused by mutations in genes coding for minor species of tRNA. The mutant tRNA molecules are then able to translate one (or more) of the nonsense codons, UAA, UAG or UGA, occurring internally in the messenger RNA. This suppression is then modified by the cytoplasmic gene [psi], suggesting that [psi] is involved in the mechanism of protein synthesis. Failure to find a linkage between [psi] and the mitochondrial genes determining erythromycin resistance and respiratory competence, coupled with the observation of a nuclear allele 'R' which interferes with the transmission of [psi] but not with that of [ERY]R or [rho]+ led to the proposal that [psi] is on a separate "extrachromosomal chromosome" (Young and Cox 1972).

The non-Mendelian mutation [URE3] changes enzymatic activities linked to nitrogen metabolism, conferring the ability to incorporate ureidosuccinic acid. It was shown that this determinant is not located on mt DNA (Lacroute 1971) and cytoduction experiments have shown that the inheritance of the determinant is not linked to the nucleus
but enters the receptor strain, which is *petite*, at the same time as the [rho]$^+$ mitochondria (Aigle and Lacroute 1975).

[TET 1] confers resistance to triethyl tin and to other trialkyl tins and triphenyl tins and is unlinked to any other mitochondrial loci (Lancashire and Griffiths 1971; Griffiths *et al*. 1975). However, evidence for its extra-mitochondrial location is still debatable.

Finally there exists in *S. cerevisiae* an extra-chromosomal species of DNA, termed 'omicron' DNA, of length 2 μm which has been shown to be of both non-nuclear and non-mitochondrial origin (Clark-Walker and Miklos 1974A) but which as yet has no specified functions. Suggestions have been made to link some of the above cytoplasmic genes to this plasmid 'omicron' DNA (Clark-Walker 1972, 1973; Griffiths *et al*. 1975). The isolation and characterization of 'omicron' DNA and its possible functions are detailed in Section 2.2 of this thesis.

(D.3) Mitochondrial genes and the *petite* mutation

The demonstration of antibiotic-resistant markers on the mitochondrial genome of yeast strains has resulted in a number of studies attempting to relate the known properties of *petite* strains to an observable change in the coding capacity of the genome, reflected in the loss or retention of antibiotic-resistance (Deutsch *et al*. 1974; Uchida and Suda 1973; Suda and Uchida 1974; Nagley *et al*. 1974; Michels *et al*. 1974; Faye *et al*. 1973; Molloy *et al*. 1975; Linnane *et al*. 1968B; Michaelis *et al*. 1973).

All studies have shown that in both spontaneously-
arisen and EtBr-induced petites, the loss of the [rho] factor is often associated with the loss of one or more of the mitochondrial genes being monitored. Further, the longer the yeast cells are exposed to EtBr the more rapid is the decrease in the number of cells that retain all mitochondrial genes. Parallel with this is a corresponding increase in the number of cells that have lost all mitochondrial genes, e.g. after 20 mins exposure to EtBr cells with genotype $\text{CAP}^R\text{ERY}^R\text{OLI}^R = 89\%$, after 75 mins. exposure to EtBr cells with genotype $\text{CAP}^O\text{ERY}^O\text{OLI}^O = 92\%$ (Deutsch et al. 1974).

Another factor revealed in the marker retention studies is that not all markers are retained to the same degree during petite induction, that is, the cytoplasmic resistance factors $\text{[OLI]}^R$, $\text{[ERY]}^R$, $\text{[CAP]}^R$ are found to be lost during EtBr treatment with differential frequencies and this is assumed to be a reflection of the linkage relationships between these factors. Hence Uchida and Suda (1973) found that $\text{[OLI]}^R$ was retained at a higher frequency than $\text{[CAP]}^R\text{[ERY]}^R$; Michels et al. (1974) found that in both primary and secondary petite clones $\text{[ERY]}^R$ was retained at a higher frequency than $\text{[OLI]}^R$; Faye et al. (1973) found that both the $\text{[CAP]}^R\text{[ERY]}^R$ determinants were lost more frequently than either the $\text{[CAP]}^R$ or $\text{[ERY]}^R$ determinants on their own, and Deutsch et al. (1974) found that whilst the retention of $\text{[CAP]}^R$ and $\text{[ERY]}^R$ were similar, the frequency of $\text{[OLI]}^R$ retention was much higher.

In the same studies, Michels et al. (1974) demonstrated that there is no relationship between suppressiveness and loss or retention of cytoplasmic markers in spontaneously-arisen petites, whilst Faye et al. (1973)
report that buoyant density changes are observed in EtBr-induced petites that retain different cytoplasmic markers. Thus all the [rho]- [CAP]O [ERY]R mt DNAs are heavier than the [rho]+ mt DNA, all [rho]- [CAP]R [ERY]O mt DNAs are lighter than the [rho]+ mt DNA and all [rho]- [CAP]R [ERY]R mt DNAs have a buoyant density similar to that of the [rho]+ mt DNA, indicating that the segment of the mitochondrial genome carrying the [ERY]R gene is heavier and richer in GC pairs than the segment carrying the [CAP]R gene (Faye et al. 1973).

It is apparent from these marker retention studies that there is no ordered loss of marker genes from the mitochondrial genome. Further, in most strains it is clear that all markers are not retained to the same degree during petite induction. However, differences exist between the retention of the [ERY]R determinant and the [OLL]R determinant in unrelated strains (compare Michels et al. 1974 and Deutsch et al. 1974).

SECTION E - COMPLEMENTATION AND THE MECHANISM OF THE PETITE MUTATION

(E.1) Present hypothesis of the mechanism of the petite mutation

Several previous hypotheses have been advanced to account for the mechanism of the petite mutation but all are found to be inadequate (Clark-Walker and Miklos 1974B). However two groups independently have proposed a mechanism that can account for this phenomenon.

The model of the wild-type mitochondrial genome of *S. cerevisiae* built up by Bernardi and co-workers, is one based on the interspersion of AT-rich and GC-rich stretches
of bases around the genome. Prunell and Bernardi (1974) estimate that the AT-rich stretches, or "spacer" regions, account for about 50% of the mitochondrial genome and have a G + C content of less than 5%. Prunell and Bernardi (1974) then propose that the extremely high spontaneous and induced rates of the petite mutation can be accounted for by a process of internal crossing-over events, involving homologous nucleotide sequences of the AT-rich or "spacer" regions.

Essentially the same model was proposed independently by Clark-Walker and Miklos (1974B), but elaborations provided explanations for polarity of recombination, suppressiveness and structural heterogeneity between mitochondrial genomes from different strains, by invoking transpositions and inversions of sequence occurring through the AT-rich sites. The postulated structural re-arrangements based on excision-insertion events, would preferentially occur at sites of partial sequence homology. The authors postulate that the AT-rich sequences in [rho]+ DNA may be the sites of partial homology that facilitate excision-insertion events leading to petite formation.

On the basis of this model, Clark-Walker and Miklos (1974B) envisage suppressiveness as the insertion of two circular molecules followed by subsequent excision from the hybrid molecule to produce defective genomes. In this mechanism, a single insertion event following homologous pairing in a [rho]- by [rho]+ cross, does not result in loss of information from the [rho]+ genome, since a partially duplicated molecule, still containing complete information, is produced. Furthermore, this molecule must
be of intermediate buoyant density, a density which depends on the relative sizes and densities of the \([\rho^-]\) and \([\rho^+]\) genomes and on the number of insertions occurring. Additionally, suppressiveness has two components; the first is related to the amount of insertion that occurs between the \([\rho^-]\) and \([\rho^+]\) genomes, and the second depends on the frequency of excisions from the partially duplicated molecules. Thus the degree of suppressiveness of a \([\rho^-]\) by a \([\rho^+]\) cross is not necessarily a reflection of the amount of genetic recombination found in that cross.

(E.2) **Complementation experiments**

An important implication of the proposed hypotheses of Bernardi and co-workers and Clark-Walker and Miklos (1974B), is that complementation between spontaneous *petites*, to give respiratory competent cells, should be possible, provided that deletions do not occur from one particular region of the mt DNA and that initially the deletions together do not exceed 50% of the combined genomes. In this respect, complementation between cytoplasmically inherited *petites* has never been reported until recently (Wright and Lederberg 1957; Jakob 1965; Roodyn and Wilkie 1967; Slonimski *et al.* 1968), despite the demonstration, genetically, that recombination between mt DNA molecules of different *petites* can occur (Michaelis *et al.* 1973), though respiratory-competent progeny were not observed as a consequence.

Successful complementation between cytoplasmic *petites* was achieved using the three criteria of (a) spontaneous *petites*, (b) those of recent origin, and (c) crosses involving all possible pairwise combinations between
the many different petite isolates of opposite mating type, (Clark-Walker and Miklos 1975A). Complementation between petites was achieved in three wild-type situations; firstly, between strains not isogenic for nuclear DNA which may not necessarily have identical mt DNA; second, between non-isogenic strains which do have identical mitochondrial genomes; and third, between strains closely related for both nuclear and mt DNA. These results show clearly that there is no unique site which is initially deleted in the first event of petite formation. Further complementation between petites from wild-type strains having the same mt DNA eliminates the possibility of each strain having its own unique site involved in [rho]+ degradation.

SECTION F - PETITE-NEGATIVE YEASTS

(F.1) Phenomenon

The majority of yeast species tested do not form cytoplasmically-inherited, respiratory deficient mutants. Out of 86 strains of yeasts tested with petite-inducing agents, 63 strains failed to give rise to viable petite colonies (Bulder 1964; De Deken 1961, 1966; Appendix II of this thesis). Bulder (1964) termed petite-positive those yeast strains that gave rise to viable, cytoplasmically-inherited petite isolates, whilst those strains that failed to yield such isolates were termed petite-negative. Petite-negative yeasts can accommodate the respiratory-deficient phenotype when the mutation is located on nuclear chromosomes, as such mutants have been isolated from three separate petite-negative yeasts (Herman and Griffin 1968; Wolf et al. 1971; Heslot et al. 1970A, B). These studies have been extended by examining thirty-nine nitrosoguanidine-
induced respiratory-deficient mutants of *Kluyveromyces lactis* 'which arose from 10,000 cells at a 10-20% survival rate'. All mutants had altered cytochrome contents and all were chromosomally-inherited (Del Guidice and Puglisi 1974). *Petite*-negative yeasts can also accommodate cytoplasmically-inherited drug resistance (Brunner *et al.* 1973).

Thus only the minority of yeast species give rise to viable cytoplasmically-inherited, respiratory-deficient mutants. A hypothesis has been advanced to account for the difference between *petite*-positive and *petite*-negative yeasts and is discussed below.

(F.2) **Hypothesis**

In proposing a hypothesis to account for the phenomenon of *petite*-negative yeast, Clark-Walker and Miklos (1974B) considered two possible explanations; the first being that the mt DNA of *petite*-negative yeasts carries a gene or genes necessary for viability; and secondly, that mt DNA in *S. cerevisiae* and other *petite*-positive yeasts has some peculiar structural properties which favour the excision-insertion process outlined previously (Section E.I).

In relation to the first proposal, it is envisaged that the mt DNA of *petite*-negative strains may well undergo excision-insertion events to produce defective genomes, but in these cases the cells containing the smaller genomes would be deficient for one or a number of mitochondrial genes necessary for survival, and hence would be inviable. It is possible that mitochondrial genomes, as illustrated by *S. cerevisiae*, may encode information necessary for the optimal growth of the yeast cell (Clark-Walker and Linnane
1967). The results of this study may be interpreted to mean that a transcriptional product of the mitochondrial genome, which is translated by cytoplasmic ribosomes, is necessary for optimal growth rate. In this case, it may be possible that petite-negative yeasts are much more, or even entirely dependent on, mitochondrial transcription products for their growth and survival. In the case of chromosomally inherited lesions leading to the respiratory-deficient phenotype in petite-negative yeasts, the mt DNA would remain intact.

In relation to the second proposal, it has been shown that the present picture of the S. cerevisiae mitochondrial genome is based on the proposal that A + T-rich sequences are interspersed with G + C-rich sequences. These A + T-rich sequences account for about 50% of the mitochondrial genome and may well provide the sites of partial homology facilitating excision-insertion and rearrangement events leading to the formation of defective genomes.

The authors envisage that there may be two components to petite-negativity, one being the existence of essential genes located on mt DNA, the loss of which leads to cellular inviability, and the second being the restrictions imposed by the actual structural features of mt DNA.

SECTION G - AIMS OF THE PRESENT STUDY

Two of the ideas formulated by Clark-Walker and Miklos (1974B) in relation to the mechanism of the petite mutation, are open to testing; these being aspects of the problems of petite-negative yeasts and suppressiveness.

In considering the problem of petite-negative yeasts,
it is postulated, as outlined above, that the mt DNA of these yeasts either possesses viability genes, the loss of which are lethal to the cell, or that the mt DNA lacks the peculiar structural features of the mt DNA of *S. cerevisiae* which permit the latter yeast to undergo *petite* formation.

In consideration of the viability gene argument, the slow growth phenomenon of *petites* of *S. cerevisiae* has been investigated in the hope that this may provide evidence that viability genes on mt DNA may be important to the growth of the cell (Chapter 1).

The second postulated explanation for *petite*-negativity involves structural features of mt DNA. This has been examined by isolating and characterizing mt DNA from diverse species of *petite*-negative yeasts (Chapter 2). In view of these findings, the examination of mt DNA has been widened to include *petite*-positive yeasts other than in the genus *Saccharomyces* (Chapter 3).

In considering the problem of suppressiveness, it is postulated, as outlined above, that this phenomenon is dependent on both the degree of insertion between the circular genomes of the wild-type and *petite* strains, and also on the degree of excision from the hybrid molecule so formed. In regard to this postulate, suppressiveness is examined by studying the interaction between a spontaneous *petite* isolate and two strains of opposite mating-type (Chapter 4).

Finally, the reasoning behind the choice of the various yeast species, both *petite*-positive and *petite*-negative, that have been employed in this study is outlined in Appendix II.
CHAPTER 1

GROWTH CHARACTERISTICS OF RESPIRATORY-DEFICIENT MUTANTS OF

*S. CEREVISIAE*

SECTION 1-1

SLOW GROWTH
Introduction

Early studies on petite mutants of *S. cerevisiae* showed that no growth occurred on a non-fermentable substrate due to the absence of a functional electron transport chain (Tavlitzki 1949; Slonimski 1949B; Ephrussi 1953). However, a second effect of the mutation, which was not so easily interpreted, was that the growth rate of petite cells on a glucose medium was reduced when compared to the wild-type (Ephrussi *et al.* 1949C; Harris 1956). The interpretation of this phenomenon was further complicated by the results of Clark-Walker and Linnane (1967), who found that chloramphenicol (CAP), which mimics the effect of the cytoplasmic mutation in rendering cells temporarily respiratory-deficient, does not produce a reduction in the growth rate of [rho]+ cells on glucose medium. Subsequently, it has been suggested that these two observations may mean that a mitochondrial transcription product is translated by cytoplasmic ribosomes (Clark-Walker and Miklos 1974B). In the case of cytoplasmic petites transcription could be eliminated, whereas in chloramphenicol-treated cells only translation is affected (Clark-Walker and Linnane 1967). These ideas were advanced by Clark-Walker and Miklos (1974B) in the context of examining the phenomenon of petite-negative yeasts.

In this respect, the previous results (Clark-Walker and Linnane 1967) were interpreted to mean that mitochondrial genomes may encode information necessary for the optimal growth of the yeast cell. Hence it may be possible that petite-negative yeasts are much more, or even entirely
dependent on, mitochondrial transcription products for their growth and survival. Although petite-negative yeasts cannot form viable, cytoplasmically-inherited, respiratory-deficient mutants, several species are known that form viable, chromosomally-inherited, respiratory-deficient mutants (Heslot et al. 1970B; Crandall 1973B; Herman and Griffin 1968; Del Guidice and Puglisi 1974; Wolf et al. 1971). In the case of chromosomally-inherited lesions leading to respiratory-deficiency in petite-negative yeasts, the mt DNA would remain intact and hence the cell would not lose the mitochondrial transcription products.

In this study, the growth rates of respiratory-deficient mutants, from a range of wild-type S. cerevisiae strains, have been determined, both in the presence and absence of chloramphenicol, in order to ascertain if the slow growth phenomenon associated with the petite mutation, reflects the presence of a gene which may be related to the postulated viability gene on the mt DNA of petite-negative yeasts.

Materials and Methods

Organisms

Four haploid and two diploid strains of S. cerevisiae were used. The haploid strains 4545, 4342, 410, X2180IA and one diploid strain M, are all described in Table I-1 (Appendix I). The other diploid strain was generated by crossing two of the above haploid strains, 4545 x 4342, and has been designated "Diploid 4545 x 4342".
Methods

The methods used are described in detail in Appendix I. Briefly, all growth analyses were determined by following the change in optical density at 640 nm of cultures growing in 50 mls of a 1% GYP medium in 125 ml conical flasks shaken at 200 rpm and maintained at 30°C in a gyrotary water-bath shaker. All optical density readings were taken on a Zeiss M4QIII/PMQII spectrophotometer, and dry weight can be related to optical density using a value of 0.019 mgs dry weight/ml, giving an optical density of 0.1 at 640 nm.

Chloramphenicol (CAP): which was a gift of Parke, Davis and Co., N.S.W., was used at a final concentration of 4 mgs/ml in the 1% GYP medium.

L(+)-threo isomer of CAP: was prepared from L(+)-threo-1-p-nitro phenyl-2-amino-1,3-propanediol and methyl dichloracetate by a published procedure (Controulis et al. 1949; Rebstock 1950), and was a gift of Dr. G. D. Clark-Walker.

Results

(A.1) Growth rates of wild-type and petite mutants of strain M both in the presence and absence of CAP

A series of spontaneously-arisen (s[rho]) and EtBr-induced (e[rho]) petites were isolated from strain M and their growth rates determined both in the presence and absence of CAP, as was the parent respiratory-competent strain. As reported before (Clark-Walker and Linnane 1967) it was found that CAP had no effect on the growth rate of either the wild-type or mutant strains (Fig. 1-1, 1-3a, 1-4a).
FIG. 1-1

Growth curves of *S. cerevisiae* strain M and an EtBr-induced petite, M e[rho]$^{-3}$, in the presence and absence of CAP in 1% GYP medium at 30°C. The curves represent:

- - ○: Strain M
- - □: Strain M in the presence of CAP (4 mgs/ml)
O - O: Strain M e[rho]$^{-3}$ both in the presence and absence of CAP (4 mgs/ml).

The doubling times of the cultures are:

(a) Strain M in the presence and absence of CAP - 74 mins.
(b) Strain M e[rho]$^{-3}$ in the presence and absence of CAP - 102 mins.

The petite strain has a growth rate of 73% of the wild-type. CAP has no effect on the growth rate of either the wild-type or petite strain M.
FIG. 1-2

Histograms showing the difference in growth rate between petite strains and the corresponding wild-type strain. The growth rate of the petite strains is expressed as a percentage of the growth rate of the corresponding wild-type strain, calculated from the ratio:

$$\frac{[\rho^+] \text{ doubling time}}{[\rho^-] \text{ doubling time}} \times 100.$$  

The figures refer to the \textit{S. cerevisiae} strains used and represent: (a) strain M, (b) strain 4545, (c) strain 410, (d) strain 4342, (e) strain X2180IA. The open boxes represent spontaneously-arising petite isolates and the solid boxes represent ethidium-bromide induced petite isolates.

FIG. 1-3

Histograms showing the effect of CAP on the wild-type strains used. The growth rate of the wild-type strain in the presence of CAP is expressed as a percentage of the growth rate of the wild-type strains in the absence of CAP calculated from:

$$\frac{[\rho^+] \text{ doubling time}}{[\rho^+] \text{ + CAP doubling time}} \times 100.$$  

The figures refer to the \textit{S. cerevisiae} strains used and represent: (a) strain M, (b) strain 4545, (c) strain 410, (d) strain 4342, (e) strain X2180IA, (f) diploid 4545 x 4342.
Number of petite strains

1-2

1-3

F

E

D

C

B

A

DIPLOID

(4545 x 4342)

X2180 IA

4342

410

4545

M

Growth rate

Growth rate

Number of petite strains

Number of determinations

60 80 100% \( \frac{\rho^+}{\rho^-} \)

60 80 100% \( \frac{\rho^+}{\rho^+ + \text{cap}} \)
FIG. 1-4

Histograms showing the effect of CAP on the growth rate of the petite strains used. The growth rate of the petite strain in the presence of CAP is expressed as a percentage of the growth rate of the same petite strain in the absence of CAP, calculated from:

\[
\frac{[\rho]^- \text{ doubling time}}{[\rho]^- + \text{CAP doubling time}} \times 100.
\]

The figures refer to the strains used, and represent: (a) strain M, (b) strain 4545, (c) strain 410, (d) strain 4342, (e) strain X2180IA. The open boxes represent spontaneously-arising petite isolates and the solid boxes represent ethidium-bromide induced petite isolates.

FIG. 1-5

Comparison of the inhibitory effect of CAP on the growth rate of both wild-type and petite strains. The growth rate of the petite strain in the presence of CAP is expressed as a percentage of the growth rate of the corresponding wild-type strain in the presence of CAP, calculated from:

\[
\frac{[\rho]^+ + \text{CAP doubling time}}{[\rho]^+ + \text{CAP doubling time}} \times 100.
\]

The figures refer to the strains used and represent: (a) strain M, (b) strain 4545, (c) strain 410, (d) strain 4342, (e) strain X2180IA. The open boxes represent spontaneously-arising petite isolates and the solid boxes represent ethidium-bromide induced petite isolates.
Number of petite strains

A. Growth rate

B. 4545

C. 410

D. 4342

E. X2180 IA

Growth rate

\[
\frac{[\rho^-]}{[\rho^-]+\text{cap}} \times 100\%
\]
The final cell yield of the wild-type strain in the 1% glucose medium in the absence of CAP was approximately three times the cell yield in the presence of CAP. The final cell yield attained by the respiratory-deficient mutants in the 1% glucose medium, in the presence or absence of CAP, was the same as that attained by the wild-type strain in the presence of CAP.

The growth rates of all *petites* were slower than that of the wild-type strain, with the growth rate of the s[rho]− strains being in the range of 55-70% (median 60%) that of the wild-type and the e[rho]− strains being in the range 75-85% (median 82%) that of the wild-type strain. These results are similar to the previous finding, which used an acriflavine-induced *petite* of strain M (Clark-Walker and Linnane 1967).

(B.1) Growth rates of other wild-type strains and their corresponding *petites*

The study was widened to establish if the results presented for strain M and its *petites* were common for all strains and also in the hope that some genetic studies could be performed. The four haploid strains employed were chosen on the basis of being the most commonly used laboratory strains.

The haploid strains, 4545, 4342, 410 and X2180IA, all doubled at varying rates but their growth rate was always slower than that of both diploid strains used (Table 1-1). The growth rates of a series of spontaneously-arisen *petites* and of EtBr-induced, neutral *petites* of each haploid strain are expressed as a percentage of the growth
### TABLE 1-1

**Doubling times of wild-type strains at 30°C**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Number of determinations</th>
<th>Doubling time (mins)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Range</td>
<td>Mean</td>
</tr>
<tr>
<td>M</td>
<td>8</td>
<td>63-75</td>
<td>69 ± 4</td>
</tr>
<tr>
<td>4545</td>
<td>19</td>
<td>72-90</td>
<td>81 ± 4</td>
</tr>
<tr>
<td>4342</td>
<td>7</td>
<td>80-87</td>
<td>83 ± 3</td>
</tr>
<tr>
<td>410</td>
<td>6</td>
<td>91-99</td>
<td>96 ± 3</td>
</tr>
<tr>
<td>x 2180IA</td>
<td>4</td>
<td>84-87</td>
<td>86 ± 2</td>
</tr>
<tr>
<td>Diploid</td>
<td>3</td>
<td>74-77</td>
<td>75 ± 1</td>
</tr>
<tr>
<td>(4545 x 4342)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
rate of the wild-type parent strain (Fig. 1-2 b-e).

In all strains studied, the cytoplasmic petites grew at a slower rate than the corresponding wild-type strain. The exponential growth rate of each series of petite isolates varied, e.g. 410 [rho]− grew at 80-90% of the wild-type whilst 4545 [rho]− grew at 70-80% of the wild-type, but the median value of each series fell in the range of 75-85% of the growth rate of the wild-type strain. Furthermore, there was no detectable difference between the growth rates of the spontaneously-arisen and EtBr-induced petites in each strain.

This result is in contrast to that obtained with strain M.

(B.2) **Effect of CAP on the growth rate of haploid s[rho]− and e[rho]− petites**

As illustrated in a previous study (Clark-Walker and Linnane 1967) and reported in this study, CAP has no effect on the initial growth rate of either the wild-type (Fig. 1-3a) or petite isolates (Fig. 1-4a) of strain M.

By contrast the growth rates of the other *S. cerevisiae* strains were affected by CAP. The reduction in growth rate varies between strains and appears to be slightly less with the petites than with the corresponding wild-type strains (Fig. 1-3 b-e and Fig. 1-4 b-e). This last point can be seen more clearly by comparing both the wild-type and petite strains grown in the presence of CAP (Fig. 1-5 a-e). If CAP has an equal effect on the growth rate of both wild-type and petite, the results obtained
should resemble closely the comparison of wild-type and petite growth rates in the absence of CAP (Fig. 1-2 a-e). With strain M, CAP has no effect on either the wild-type or petite strains and hence, as expected, Fig. 1-5 a resembles closely Fig. 1-2 a. With all the other strains, the reduction in growth rate brought about by the presence of CAP in the medium is greater for the wild-type strains (about 5-10% more) than the reduction seen in the growth rate of the corresponding petites (Figs. 1-5 b-e compared to Figs. 1-2 b-e).

This differential response to CAP observed between strain M and the other *S. cerevisiae* strains may reflect a difference between haploids and diploids. To test this, a series of diploids were generated by crossing two of the studied haploid strains, 4545 (a) and 4342 (a), and their response to CAP studied. It was found that the exponential growth rates of the generated diploids were reduced by the presence of CAP in the medium to about 80% of the rate in the absence of the drug (Fig. 1-3 f). Thus it appears that the apparent lack of effect of CAP on the growth rate of strain M is not due to the diploid nature of the strain.

(C.1) **Effect of the L(+) threo isomer of CAP**

An important control is to determine if the reduction in growth rate brought about by CAP is due specifically to the action of CAP or is due to a non-specific effect of the antibiotic at high concentration (4 mgs/ml). Therefore, the effect of the non-biologically active isomer of CAP, L(+) threo CAP, was tested. The
exponential growth rates of strains M [rho]$^+$, 4545 [rho]$^+$ and 4545 e[rho]$^-$ in the presence and absence of both CAP isomers are tabulated (Table 1-2). Neither isomer has an effect on strain M, but with both the wild-type and the EtBr-induced petite of strain 4545, the non-active isomer of CAP has a slight effect on the growth rate. The reduction in growth rate brought about by the non-active isomer of CAP is not as great as that produced by the active isomer of CAP. The reduction caused by the non-active form is about 10% in both strains, whilst the reduction caused by the active isomer is slightly greater in the wild-type strain, 23%, than it is in the petite strains 17%.

(D.1) **Ascospores from strain M**

Haploids of strain M were sought for genetical studies of both the slow-growth phenomenon and the lack of the CAP effect shown by the wild-type strain. However, after many attempts at sporulation and dissection using two different stocks of strain M, it proved impossible to obtain asci yielding four viable ascospores. Most asci contained only one or two ascospores with a few having three.

(E.1) **Growth analysis of a nuclear RD mutant**

A chromosomally-inherited, respiratory-deficient mutant was isolated from strain X2180IA by Dr. G. D. Clark-Walker, as a spontaneously-arising mutant in the course of doing a cytoplasmic petite complementation experiment. Subsequently, it was identified as a possible nuclear mutant by complementation when crossed to a known neutral petite, 4545 e[rho]$^-$, of opposite mating type. The respiratory-
TABLE 1-2

Effect of L(+) threo isomer of chloramphenicol on strains used

<table>
<thead>
<tr>
<th>Strain</th>
<th>% Doubling time of wild-type</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) M [rho]$^+$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100% (70 mins)</td>
</tr>
<tr>
<td>+ CAP</td>
<td>99%</td>
</tr>
<tr>
<td>+ L(+) threo CAP</td>
<td>100%</td>
</tr>
<tr>
<td>(B) 4545 [rho]$^+$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100% (82 mins)</td>
</tr>
<tr>
<td>+ CAP</td>
<td>77%</td>
</tr>
<tr>
<td>+ L(+) threo CAP</td>
<td>93%</td>
</tr>
<tr>
<td>(C) 4545 e[rho]$^-$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100% (103 mins)</td>
</tr>
<tr>
<td>+ CAP</td>
<td>83%</td>
</tr>
<tr>
<td>+ L(+) threo CAP</td>
<td>91%</td>
</tr>
</tbody>
</table>
sufficient diploids formed with the neutral petite were sporulated and the mutant was shown to be a chromosomally-inherited, respiratory-deficient mutant by tetrad dissection. Of six dissected asci, all gave four viable ascospores which segregated both 2:2 for growth/non growth on glycerol plates (GLYYP) and 2:2 for large/small colonies on a glucose plate (GYP), with the large colony ascospores growing on GLYYP, whilst the small colony ascospores failed to do so. The other nuclear markers also segregated 2:2 in the ascospores.

A complete set of 4 ascospores from a single ascus were used in the subsequent growth analysis. The growth characteristics of the wild-type strain, X2180IA, and an EtBr-induced petite have already been presented (Table 1-1, Figs. 1-2 e to 1-5 e).

For this analysis two double petite mutants, pet⁻ e[rho]°, were isolated from each respiratory-deficient ascospore by EtBr-induction and subsequent analysis showed the mutants to have lost all detectable mt DNA (results not presented). Growth characteristics of the two respiratory-sufficient ascospores, pet⁺ [rho]⁺, the two respiratory-deficient ascospores, pet⁻ [rho]⁺ and the four double petites, pet⁻ e[rho]°, were determined and are presented (Fig. 1-6 a, b, c).

With the pet⁻ [rho]⁺ ascospores, the percentage of cytoplasmic petites in each culture was determined before and after each growth analysis by crossing the culture with a known neutral petite strain of opposite mating type. The
FIG. 1-6

Growth histograms of the nuclear petite isolated from strain X2180IA. The boxes correspond to:

open boxes: 2 respiratory-sufficient ascospores, i.e. \( \text{pet}^+ \ [\text{rho}]^+ \).

hatched boxes: 2 chromosomally-inherited, respiratory-deficient ascospores, i.e. \( \text{pet}^- \ [\text{rho}]^+ \).

solid boxes: 4 EtBr-induced double petites, 2 from each of the \( \text{pet}^- \) ascospores, i.e. \( \text{pet}^- \ [\text{rho}]^0 \).

The histograms represent:

(a) The growth rate of the isolates expressed as a percentage of the wild-type growth rate, i.e.

\[
\frac{\text{doubling time of wild-type}}{\text{doubling time of isolates}} \times 100.
\]

(b) The effect of CAP on the isolate, i.e. the growth rate of the isolate in the presence of CAP expressed as a percentage of the growth rate in the absence of CAP, i.e.

\[
\frac{\text{doubling time of isolate}}{\text{doubling time of isolate + CAP}} \times 100.
\]

(c) A comparison of the growth rates of both the isolate and the wild-type in the presence of CAP, i.e.

\[
\frac{\text{doubling time of wild-type + CAP}}{\text{doubling time of isolate + CAP}} \times 100.
\]
Number of isolates

C  Wild-type + cap
    Strain + cap

B  Strain
    Strain + cap

A  Wild-type
    Strain

Growth rate

60  80  100  120%
amount of cytoplasmic petites varied from 1.6 to 2.8% in all cases, compared to the spontaneous petite frequency of 1.1% in the wild-type strain. This small percentage of [rho]^- colonies would have a minimal effect on the growth rate of the pet^- [rho]^+ strains.

It was found that all respiratory-deficient mutants of strain X2180IA, both chromosomally- and cytoplasmically-inherited, grew slower than the wild-type strain, at about 70-80% of the wild-type growth rate (Fig. 1-2 e, 1-6 a). Furthermore, there was no detectable difference in growth rate between the pet^-, the [rho]^- or the pet^- [rho]^- mutants.

(E.2) Effect of CAP on the growth rate of a nuclear RD mutant

The effects of CAP on the growth rates of the respiratory-sufficient and respiratory-deficient isolates from the nuclear RD mutant were studied.

It was found that the exponential growth rates of all the [rho]^+ isolates, that is the pet^[rho]^+ and pet^- [rho]^+ ascospores, in the presence of CAP were reduced to about 85% of their growth rate in the absence of CAP (Fig. 1-6 b), resembling the effect of CAP on the wild-type strain (Fig. 1-3 e).

In contrast, the reduction in growth rate of the pet^- [rho]^0 strains in the presence of CAP was not as great, being about 92% of the growth rate in the absence of CAP (Fig. 1-6 b). This situation resembles the effect of CAP on the EtBr-induced cytoplasmic petite (Fig. 1-4 e). Again the reduction in growth rate brought
about by the presence of CAP is not as great with the [rho]$^0$ strains as it is with the [rho]$^+$ strains. This effect can be seen more clearly by comparing Figs. 1-6 c and 1-6 a.

In conclusion it can be seen that CAP reduced the growth rate of both chromosomally- and cytoplasmically-inherited respiratory-deficient mutants of strain X2180IA.

Discussion

All cytoplasmic petites of S. cerevisiae grew at a slower rate than the corresponding wild-type parent strains. This reduction in growth rate varies between strains but usually petites grew at 70-80% the rate of the wild-type. Furthermore, there was no difference in growth rate between spontaneously-arisen and EtBr-induced petites of the same strain, except possibly in the case of strain M.

A nuclear RD mutant from strain X2180IA also shows a reduction in growth rate to 75% the rate of the wild-type strain. Superposition of the petite mutation on the nuclear-coded, respiratory-deficient genotype has no further effect on the growth rate of the nuclear mutant. It is known that chromosomally-inherited, respiratory-deficient mutants that complement when crossed, indicating that single genes are involved, can have the same pleiotropic effects, that is inhibition of cytochromes aa$_3$ and b synthesis (Sherman 1963). However, it is not known whether the slow-growth character found in association with this mutant is common to all other nuclear-coded mutants. Further, the question also arises of whether the trans-
cription of the mitochondrial genome still occurs in the nuclear RD mutants and/or in the \( [\text{rho}]^- \) *petites*. The fact that the EtBr-induced \( \text{pet}^- [\text{rho}]^0 \) *petites* show no difference in growth rate to the \( \text{pet}^- [\text{rho}]^+ \) mutants could argue that transcription is shut off in both cases. However, the results of Lamb and Rojanapo (1973) and Cohen *et al.* (1972) suggest that mitochondrial transcription still occurs in \( [\text{rho}]^- \) mutants and recently protein products have been translated from the mt RNA of a *petite* in an *Escherichia coli* cell-free protein synthesising system (Halbreich *et al.* 1975) showing that *petites* still retain the capacity to transcribe their remaining mt DNA.

In view of these results, it appears highly unlikely that mitochondrial transcription is shut off in the nuclear respiratory deficient mutant. Hence, the reduction in growth rate of both the \( \text{pet}^- [\text{rho}]^0 \) and \( \text{pet}^- [\text{rho}]^+ \) mutants would negate the transcription product argument (Clark-Walker and Miklos 1974B) for optimal growth of the cell. However, support for the transcription product hypothesis would appear to come from the reported complementation among cytoplasmic mutants of *Neurospora crassa* (Bertrand and Pittenger 1972), where the observed functional complementation implies that mitochondrial gene products are exported to the cytoplasm and may be important in determining the rate of growth of the organism.

The experiments involving CAP proved to be a digression from the main purpose of this study. The lack of effect of CAP on the exponential growth of strain M and its
petites was the same as reported previously (Clark-Walker and Linnane 1967). This showed that in strain M, CAP which inhibits mitochondrial protein synthesis and mimics the effect of the petite mutation in rendering cells temporarily respiratory-deficient (Clark-Walker and Linnane 1967), does not cause a reduction in initial growth rate due to glycolysis. With the other strains studied, what appears to be happening is that if CAP lowers the growth rate of the wild-type strain, then the antibiotic also lowers the growth rate of the corresponding petite strains, although not to such an extent.

This effect of CAP appears to have two components, firstly a non-specific reduction in growth rate brought about by both the active D(-) threo and the non-active L(+) threo isomers of CAP, due probably to the presence of the drug at such a high concentration. The second component is a specific reduction in growth rate of the [rho]+ strains due to the effect of the active D(-) threo isomer on the mitochondrial protein synthesising system. This second component may be due to a small contribution of partially glucose derepressed mitochondria to the growth rate; that is these strains may not be as highly glucose repressed as strain M.

The results using CAP are further complicated by the reduction in growth rate that the active isomer brings about in petite cells that are reported to already lack a functional protein synthesising system (Schatz and Saltzgaber 1969). This naturally raises the question of whether all mitochondrial protein synthesis is inhibited in
petite cells, as reported by Schatz and similarly, whether all mitochondrial protein synthesis is inhibited in nuclear respiratory-deficient mutants. Of relevance is the observation that a small amount of translation still occurs in CAP inhibited cells of strain M as revealed by the fact that amino acid incorporation is never inhibited to 100% in such cells (Lamb et al. 1968). This may mean that although the CAP inhibited cells of strain M are unable to grow on a non-fermentable substrate, sufficient translation may still occur to allow the cell to carry out some other function in the mitochondrion.

Of possible significance to this idea are reports that the mitochondria of S. cerevisiae must carry out a function necessary to the survival of the cell, even though the cell is unable to grow on a non-fermentable substrate. For example, there exist nuclear mutations in S. cerevisiae which render the cells unable to utilise non-fermentable substrates. Three such mutants, op1, pet 936 and Z1 x 1, are lethal if the petite mutation is superimposed on the nuclear background, that is if the mt DNA is eliminated in these nuclear mutants. These three mutants, op1 (with an affected adenine nucleotide translocase (Kolarov et al. 1972)), pet 936 (lacking mitochondrial adenosine triphosphatase (Ebner and Schatz 1973) and Z1 x 1 (of unknown function (Subik 1974)) in effect render these S. cerevisiae strains petite-negative as the mutant strains are unable to form viable cytoplasmic petites. In relation to these studies, the question is raised as to whether CAP also renders the mutant cells
inviable or whether the inhibition of translation has no effect.

Additional evidence for the presence of vital mitochondrial functions comes from reports that a mitochondrial ATP translocase is necessary for continued cell growth (Subik et al. 1972) and that bongkrekic acid inhibition of this translocase in either [rho]− or petite-negative cells leads to cell arrest (Subik et al. 1974). These reports emphasise the need of active ATP translocation into and out of mitochondria in [rho]− and petite-negative cells.

In consideration of all this data, a new concept can be proposed about the presence of viability genes on the mitochondrial genome. It can be postulated that certain vital mitochondrial functions are associated with yeast mt DNA. In *S. cerevisiae*, the coding for these vital mitochondrial functions is shared between the nuclear and cytoplasmic genes, whereas petite-negative yeasts may rely solely on mitochondrial genes for these functions. In this case, in *S. cerevisiae* loss of one set of genes may be inhibitory, as evidenced by the slow-growth character, but loss of both sets of genes would be lethal, as evidenced by the nuclear mutants *op*1, *pet*936 and Z1 x 1. On the other hand, in petite-negative yeasts loss of the genes from the mitochondrial genome would be lethal, but nuclear-coded, respiratory-deficient mutants would still retain an intact mitochondrial genome and hence the vital functions would not be lost.
In conclusion, the results presented show that the slow-growth phenomenon of \textit{petite} strains is a characteristic of the mutation. Although a new concept is proposed for this phenomenon, the question of why it is that respiratory-deficient mutants grow at a slower rate than the corresponding wild-type strain in a glucose medium, still remains to be answered.
SECTION 1-2

ISOLATION AND CHARACTERIZATION OF FASTER-GROWING STRAINS OF RESPIRATORY DEFICIENT MUTANTS
Introduction

In the previous Section, it has been shown that the slow-growth phenotype is a characteristic of all cytoplasmic petite mutants and the single isolate of a nuclear respiratory-deficient mutant of *S. cerevisiae*.

During a mitochondrial genome decay experiment, performed by Dr. G. D. Clark-Walker, it was noted that a single colony type overtook the culture. Characterization of this respiratory-deficient mutant showed it to be a faster-growing variant which had lost the slow-growth phenotype and which now had a doubling-time equal to that of the wild-type strain.

A study was therefore initiated into faster growing variants in the hope that this might clarify the nature of the slow-growth lesion.

Materials and Methods

Strains

The yeast strains used were spontaneous petite isolates of the *S. cerevisiae* strains used previously. These wild-type strains were: 410, 4545 and 4545 ERY\(^R\), a spontaneous, erythromycin-resistant isolate of strain 4545, obtained by growing wild-type cells on gly YP plates containing 2.5 mgs/ml erythromycin. This last strain was isolated by Dr. G. D. Clark-Walker.

Methods

Growth analysis conditions are the same as before and are detailed in Appendix I. The methods for repeated sub-culture of cells, calculation of generation number,
determination of suppressiveness and methods for preparation of closed-circular DNA are detailed in Appendix I.

Results

(A.1) A faster-growing \([\text{rho}]^-\) isolate from strain 4545 \(\text{ERY}^R\)

The first faster-growing \([\text{rho}]^-\) strain was isolated during a mitochondrial genome decay experiment performed by Dr. G. D. Clark-Walker. The purpose of this study was to monitor the decay of the erythromycin-resistant (\(\text{ERY}^R\)) gene in a group of spontaneous \([\text{rho}]^-\) isolates. In the experiment, 48 petite colonies were picked after 72 hours growth on GGYP plates and inoculated into 50 mls of 2\% GYP. During the period of continuous sub-culture, the retention of the genetic marker on the mitochondrial genome was monitored by testing individual colonies arising from plating the culture onto GGYP plates. The decay of this marker as the culture progressed, is shown (Fig. 1-7). Over the first 50 generations (37-87 generations after the start) the resistance retention is constant and on GGYP plates the majority of the colonies are the same size. After 135 generations, two distinct sets of colonies were apparent on GGYP plates. One set was larger than the other and none of its isolates retained any erythromycin resistance. Isolates of the other small set retained a level of ERY resistance slightly below that previously found. After 222 generations, only one type of petite mutant was present on the GGYP plates. This type consisted of large colonies, none of which retained erythromycin resistance.
FIG. 1-7

Erythromycin resistance retention in *petites* from strain 4545 [rho]+ ERY\textsuperscript{R}. The figure represents the percentage of *petite* isolates which retained erythromycin resistance when picked at various time intervals after the start (0 time) of the continuous culture. Of the original 48 *petites* used from strain 4545 [rho]+ ERY\textsuperscript{R}, the erythromycin retention was 60%. The points are given by the table:

<table>
<thead>
<tr>
<th>Number of generations after start of continuous culture</th>
<th>Number of <em>petites</em> retaining ERY\textsuperscript{R}</th>
<th>% Resistance retention</th>
</tr>
</thead>
<tbody>
<tr>
<td>37.7</td>
<td>9/47</td>
<td>19.1</td>
</tr>
<tr>
<td>87.2</td>
<td>10/48</td>
<td>20.8</td>
</tr>
<tr>
<td>135.3</td>
<td>0/30 (large)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>5/32 (small)</td>
<td>15.6</td>
</tr>
<tr>
<td></td>
<td>5/64</td>
<td>8.1</td>
</tr>
<tr>
<td>222.1</td>
<td>0/48 (large)</td>
<td>0</td>
</tr>
</tbody>
</table>

The mean number of generations in a 24-hour period was 12.4 and the *petite* frequency of the wild-type strain was 2.95%.
% of colonies retaining ery resistance

Number of generations after start of continuous culture
Ten of the colonies arising after 222 generations were selected for further study. It was found firstly, that all ten isolates were faster-growing \([\text{rho}]^-\) colonies with doubling times between 99-100% that of the wild-type strain; secondly, that the suppressiveness of all ten isolates when crossed to strain 4342 fell between 84-89%; and thirdly, that the buoyant density of the mt DNA of the four colonies tested was between 1.684 and 1.685 g/cm\(^3\) (Table 1-3A), compared to the wild-type mt DNA buoyant density of 1.685 g/cm\(^3\). Hence it would appear that all ten colonies have a common origin.

Subsequently, the growth rate of the faster-growing \([\text{rho}]^-\) isolates was monitored at weekly intervals for 3 weeks, during which time the parent cultures were stored at 4°C on GYP slopes. It was found that for the first two weeks, the growth rate of sub-cultures were the same as that of the original faster-growing isolate, but by the third week the growth rate was reduced to 80%. This corresponds to the rate obtained with original spontaneous petite isolates from the parent strain 4545 (Fig. 1-2 b). Hence, the faster-growth characteristic is not stable during storage at 4°C.

These findings of the acquisition of faster-growth and its subsequent loss, were most surprising and interesting. Therefore, this study was extended to determine if these results were a general phenomenon and whether it is related to mt DNA, as the coincident loss of the erythromycin resistance and appearance of the faster growing variant might suggest.
TABLE 1-3

Characteristics of petite strains isolated by repeated sub-culturing of 'normal' petites

<table>
<thead>
<tr>
<th>Petite strain</th>
<th>Number of generations of continuous sub-culture</th>
<th>Growth rate (% growth rate of wild-type strain)</th>
<th>Buoyant density of mt DNA (g/cm³)</th>
<th>Suppressiveness (%) crossed to strain 4342</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4545 f[rho]⁻⁸</td>
<td>222</td>
<td>100%</td>
<td>1.685</td>
<td>85%</td>
</tr>
<tr>
<td>4545 f[rho]⁻¹⁰</td>
<td>222</td>
<td>100%</td>
<td>1.684</td>
<td>89%</td>
</tr>
<tr>
<td>B.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4545 f[rho]⁻¹</td>
<td>222</td>
<td>97%</td>
<td>None detectable</td>
<td>12%</td>
</tr>
<tr>
<td>4545 f[rho]⁻²</td>
<td>222</td>
<td>99%</td>
<td>None detectable</td>
<td>9%</td>
</tr>
<tr>
<td>4545 f[rho]⁻₃</td>
<td>222</td>
<td>96%</td>
<td>None detectable</td>
<td>11%</td>
</tr>
<tr>
<td>4545 f[rho]⁻₄</td>
<td>222</td>
<td>95%</td>
<td>None detectable</td>
<td>11%</td>
</tr>
<tr>
<td>4545 f[rho]⁻₅</td>
<td>222</td>
<td>101%</td>
<td>1.685</td>
<td>66%</td>
</tr>
<tr>
<td>4545 f[rho]⁻₆</td>
<td>222</td>
<td>98%</td>
<td>None detectable</td>
<td>11%</td>
</tr>
<tr>
<td>4545 f[rho]⁻₇</td>
<td>222</td>
<td>98%</td>
<td>None detectable</td>
<td>9%</td>
</tr>
<tr>
<td>4545 f[rho]⁻₈</td>
<td>222</td>
<td>91%</td>
<td>None detectable</td>
<td>85%</td>
</tr>
<tr>
<td>4545 f[rho]⁻₉</td>
<td>222</td>
<td>98%</td>
<td>None detectable</td>
<td>96%</td>
</tr>
<tr>
<td>4545 f[rho]⁻₁₀</td>
<td>222</td>
<td>99%</td>
<td>None detectable</td>
<td>87%</td>
</tr>
<tr>
<td>C.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>410 f[rho]⁻¹</td>
<td>154</td>
<td>100%</td>
<td>None detectable</td>
<td>1%</td>
</tr>
<tr>
<td>410 f[rho]⁻₄</td>
<td>154</td>
<td>100%</td>
<td>None detectable</td>
<td>1%</td>
</tr>
</tbody>
</table>
(B.1) Faster-growing \([\text{rho}]^-\) isolates from strain 4545

In this second study, the parent strain, 4545, of the previous spontaneous mutant 4545 ERY\(^R\) was employed. It was decided to isolate a faster-growing variant from strain 4545 in order to determine three things; firstly, to ensure that the phenomenon is repeatable; secondly, to study the mt DNA of the faster-growing variant, as previously the faster-growing variant from strain 4545 ERY\(^R\) had retained its mt DNA; and thirdly, to study the composition of the 'omicron' \((\text{O})\) DNA of the faster-growing variant. There occurs another cytoplasmic DNA species in the \(S. \text{cerevisiae}\) cell termed 'omicron' DNA (Clark-Walker 1972) and a change in composition of this DNA had been reported in a copper-resistant mutant from an ethidium-bromide induced neutral petite from strain 4545 (Clark-Walker and Miklos, 1975B). Hence a correlation was sought between the faster-growth phenomenon and a change in the composition of the \(\text{O}\) DNA.

The same basic method was employed to isolate faster-growing petite variants from strain 4545. In this case, 29 separate petite colonies were inoculated into the same 50 ml of 2% GYP. The incubation mixture was sub-cultured every day for 21 days and a aliquot plated onto GGYP plates after 7, 14 and 21 days repeated sub-culturing; the colony morphology was observed (on the GGYP plates) after incubation of the plates at 30°C.

After 70 generations repeated sub-culture, most colonies were the same size on GGYP plates, but some were
much smaller. After 145 generations growth, two distinct types of colony, larger and smaller, were apparent on the GGYP plates. Finally, after 220 generations, there were again two distinct sets of colonies on the GGYP plates. Both types of colony were the same size but they differed in that the majority (88%) were more red pigmented than the minority (12%).

Subsequently, ten colonies of each colour type were picked and their growth rates determined. It was found that whilst the red pigmented series had growth rates of between 89-93% (median 91%) that of the wild-type, the less pigmented (paler) series had growth rates of 91-101% (median 98%) that of the wild-type strain. Hence it would appear that the paler series were faster-growing petite isolates, which had not yet completely taken over the culture, as seen previously (Section 1-2A).

(B.2) Suppressiveness and mt DNA of the faster growing variants

The degree of suppressiveness of each of the faster-growing petite isolates was determined when crossed to strain 4342 (Table 1-3b). From the suppressiveness results, there appears to be three different cell genotypes; firstly, those isolates having low suppressiveness of about 10% (4545 f[rho]^{-1,2,3,4,6,7}) (where f stands for faster-growth); secondly, those isolates of intermediate suppressiveness, 4545 f[rho]^{-5}, and thirdly, those isolates of high suppressiveness, 4545 f[rho]^{-8,9,10}. In addition, one isolate, 4545 f[rho]^{-8}, had a consistently slower growth rate than any of the others.
Thus four faster-growing petite isolates were chosen as representative of the different suppressiveness types present. These isolates were 4545 f[rho]^{-2} and -7 (both 9% suppressive), f[rho]^{-5} (66% suppressive) and f[rho]^{-8} (85% suppressive). When mitochondria-enriched pellets from each of these faster growing petite isolates are centrifuged to equilibrium on a CsCl-EtBr gradient, two upper linear DNA bands and a lower, closed-circular DNA band are visible under UV light for isolates 4545 f[rho]^{-5} and -8, whilst only a single upper band and a lower, closed-circular band are seen with isolates 4545 f[rho]^{-2} and -7.

For each isolate, two DNA fractions are removed by side-puncture of the polyallomer tube. The first fraction, termed 'the top DNA band' comprises the uppermost band of the linear DNA part of the gradient, together with some of the lower, main band DNA. The second fraction, termed 'the closed-circular DNA band' comprises the lower closed-circular DNA band visible in the CsCl-EtBr gradients.

(B.3) Buoyant density analysis of both DNA bands

After EtBr extraction and dialysis, both the top DNA band and the closed-circular DNA band from each isolate were analysed by analytical ultracentrifugation (Fig. 1-8). For buoyant density determinations, Micrococcus luteus DNA was added as a marker.

The two faster-growing isolates, 4545 f[rho]^{-5} and -8 had two DNA components of buoyant densities 1.701 and 1.685 g/cm³ in both their top DNA band and also in their closed-circular DNA band (Fig. 1-8 A, B and C, D). The
FIG. 1-8

Uviscan traces of CsCl-buoyant density gradients in the analytical ultracentrifuge. The traces correspond to linear DNA bands (A, C, E, G) and the closed-circular DNA band (B, D, F, H) from mitochondrial-enriched fractions of faster-growing [rho]− variants isolated from strain 4545. The strains portrayed are, respectively: (A, B) 4545 f[rho]−5, (C, D) 4545 f[rho]−8, (E, F) 4545 f[rho]−2, (G, H) 4545 f[rho]−7. Note the lack of any detectable mt DNA band (buoyant density 1.685 g/cm³ or less) in either of the latter two petites. DNA from Micrococcus luteus at a buoyant density of 1.731 g/cm³ is a marker.
other two faster-growing isolates, 4545 \( f[rho]^{-2} \) and \( -7 \), had only a single DNA component of buoyant density 1.701 g/cm\(^3\) in both their top DNA band and in their closed-circular DNA band (Fig. 1-8, E, F, and G, H).

This lack of detectable mt DNA in either of the isolates 4545 \( f[rho]^{-2} \) and \( -7 \) correlates with the low level of suppressiveness of these strains when crossed to strain 4342 (Table 1-3 b). However, it should be noted that the level of suppressiveness seen (9%) is above the background level of 1% seen in the wild-type cross 4545 [rho]+ x 4342 [rho]+, or the 2% level seen in the cross 4545 e[rho]- x 4342 [rho]+.

(B.4) **Electron microscope analysis of the closed-circular DNA band**

The closed-circular DNA band from each faster-growing isolate was analysed by electron microscopy. In each case the analysis revealed the presence of a large number of small, supercoiled molecules of various sizes, together with some relaxed forms. Larger molecules were also present in both relaxed and supercoiled form (Fig. 1-9).

The length distribution of relaxed molecules seen in isolate 4545 \( f[rho]^{-2} \) (Figs. 1-9.1, 1-10c) showed that the smallest DNA molecule present, having a monomer length of 1.94 \( \mu m \), and its oligomeric forms of lengths 3.9 \( \mu m \) and 5.7 \( \mu m \), accounted for 88% of the total number of relaxed DNA molecules viewed. A second DNA species of mean length 2.83 \( \mu m \) represented 9% of the total number of relaxed DNA molecules. The other 3% of relaxed DNA molecules are large in size and not easily attributable to
FIG. 1-9

DNA molecules from the closed-circular DNA bands from the faster-growing [rho]^- variants of strain 4545. The pictures show both relaxed and supercoiled molecules from:

(1) strain 4545 f[rho]^-2, with molecules of both the omicron DNA components of sizes 2.0 and 2.8 μm.

(2) strain 4545 f[rho]^-8 with molecules of both omicron DNA, 1.9 and 3.7 μm, and of mt DNA, 1.5 and 4.6 μm.

(3) strain 4545 f[rho]^-5, with molecules of both omicron DNA, 1.9 and 5.7 μm, and of mt DNA, 0.9 and 0.5 μm. The large molecule is of length 13.0 μm.

The figures are at a magnification of 30,000. The bar in diagram (2) represents 1 μm.
FIG. 1-10

Length distribution of the relaxed circular DNA molecules seen in the electron microscope analyses of the closed-circular DNA bands isolated from three faster-growing [rho]− variants of strain 4545. The figures represent:

A. 4545 f[rho]−8 (mt DNA buoyant density 1.685 g/cm³) closed-circular DNA band analysed on the HUIIE electron microscope (mag. 9,500). The total number of molecules measured = 266. Two distinct DNA species are present, indicated by I and II.

Peak I: 
- n = 85, l = 1.46 ± 0.07 μm,
- dimers: n = 40, l = 2.87 ± 0.14 μm,
- trimers: n = 6, l = 4.40 ± 0.13 μm,

these represent 49.3% of the total circular DNA viewed.

Peak II: 
- n = 111, l = 1.84 ± 0.07 μm,
- dimers: n = 14, l = 3.74 ± 0.16 μm
- trimers: n = 6, l = 5.39 ± 0.13 μm

this 'o' DNA represents 49.3% of the circular DNA viewed.

B. 4545 f[rho]−5 (mt DNA buoyant density 1.685 g/cm³) closed-circular DNA band analysed on the JEM 100C electron microscope (mag. 11,200). Total number of molecules measured = 322. Three distinct DNA species are present, indicated by I, II, III.

Peak I: 
- n = 35, l = 0.55 ± 0.08 μm,
- dimers: n = 3, l = 1.17 ± 0.03 μm
- trimers: n = 7, l = 1.51 ± 0.04 μm

these represent 14% of the total circular DNA molecules viewed.
FIG. 1-10 (Cont.)

Peak II: \(n = 143, \ l = 0.85 \pm 0.06 \ \mu m,\)

\[\text{dimers } n = 26, \ l = 1.69 \pm 0.07 \ \mu m,\]
\[\text{trimers } n = 5, \ l = 2.56 \pm 0.07 \ \mu m,\]

these represent 54% of the total circular DNA molecules viewed.

Peak III: \(n = 66, \ l = 2.00 \pm 0.07 \ \mu m,\)

\[\text{dimers } n = 3, \ l = 3.75 \pm 0.07 \ \mu m,\]
\[\text{trimers } n = 13, \ l = 5.6 \pm 0.08 \ \mu m,\]

these represent 25% of the total circular DNA molecules viewed.

C. 4545 \(f[\rho]^{-2}\) (no detectable mt DNA) closed-circular band analysed on the JEM 100C electron microscopes (mag. 11,200). Total number of molecules measured = 143. A single DNA species, omicron DNA, is present, which has two components I, II.

Peak I: \(n = 110, \ l = 1.94 \pm 0.06 \ \mu m,\)

\[\text{dimers } n = 11, \ l = 3.92 \pm 0.07 \ \mu m,\]
\[\text{trimers } n = 5, \ l = 5.71 \pm 0.04 \ \mu m,\]

these represent 88% of the total circular DNA molecules viewed.

Peak II: \(n = 13, \ l = 2.83 \pm 0.06 \ \mu m,\)

\[\text{dimers } \text{obscured by above trimers}\]
\[\text{trimer } n = 1, \ l = 8.46 \ \mu m\]

these represent 10% of the total circular DNA molecules viewed.
either DNA species. This DNA profile is similar to that reported previously for the EtBr-induced neutral petite 4545 e[rho]− of the same wild-type strain (Clark-Walker 1973) and termed 'omicron' (o) DNA. A similar profile was also seen with the other faster-growing isolate 4545 f[rho]−7 (not shown here).

Length distribution analysis of the closed-circular DNA band from isolate 4545 f[rho]−8 revealed the presence of two distinct DNA species and their corresponding oligomeric forms (Fig.1-9.2, 1-10a). The smaller DNA species, of monomer length 1.45 μm, and molecules of length 2.9 μm and 4.4 μm, sizes which correspond to dimers and trimers of the monomer, represent 49% of the total relaxed circular DNA molecules viewed. The second DNA species, the 'omicron' DNA component, of monomer length 1.84 μm and its dimeric and trimeric forms, of lengths 3.7 μm and 5.4 μm respectively, represent 49% of the total relaxed circular DNA molecules viewed. The second o DNA species of 2.8 μm normally seen in the neutral [rho]− strain is here masked by the dimeric form of the 1.45 μm circles. The smaller circular DNA of length 1.45 μm present in strain 4545 f[rho]−8 can then be correlated or equated with the buoyant density component of 1.685 g/cm³ seen in the analytical ultracentrifuge traces of the closed-circular DNA band.

The length distribution analysis of the closed-circular DNA band from isolate 4545 f[rho]−5 showed the presence of three distinct DNA species and their oligomeric forms (Figs.1-9.3, 1-10b). The first DNA species of monomer length 0.55 μm and molecules of length 1.15 μm
and 1.5 μm, corresponding to dimeric and trimeric oligomers respectively, accounted for 14% of the total relaxed circular DNA molecules. The second DNA species of monomer length 0.85 μm and molecules of length 1.7 μm and 2.56 μm, corresponding to dimeric and trimeric oligomers respectively, accounted for 54% of the total relaxed circular DNA molecules. The third DNA species was again DNA of monomer length 2.0 μm and together with molecules of length 3.8 μm and 5.6 μm, corresponding to dimeric and trimeric oligomers respectively, accounted for 25% of the total relaxed circular DNA molecules. Again the second DNA species of length 2.83 μm normally seen in the neutral [rho]− strains is masked.

These three DNA species and their oligomeric forms accounted for 93% of the total number of relaxed circular DNA molecules. Larger molecules, of lengths from 6-21 μm, accounted for the other 7% of relaxed circular DNA molecules.

Again, by comparing the buoyant density profiles and length distribution analyses seen in the electron microscope of the closed-circular DNA bands of the neutral 4545 f[rho]−2 and of isolate 4545 f[rho]−5, it is apparent that the mitochondrial DNA component of buoyant density 1.685 g/cm³ in the latter strain is a composite of two separate DNA species with monomer lengths of 0.5 μm and 0.8 μm, present in a ratio of approximately 1:4.

(B.5) Summary

In this second repeated sub-culture experiment performed over a period of 21 days with [rho]− isolates of strain 4545, the final growth culture did not
contain a single group of *petites* with identical phenotypes as seen previously (Section 1-2A) but instead contained a variety of types. Firstly, the faster-growing [*rho]* variants were in the minority in the final mixture and secondly, three different mitochondrial phenotypes can be distinguished in the faster-growing variants on the basis of the presence or absence of mt DNA and on the size of the circular molecules present in the 1.685 g/cm³ buoyant density component. This result is different from that obtained with the first repeated sub-culture experiment (Section 1-2A) where a single strain established itself, but might be due to an early termination of the repeated sub-culturing. It is felt that a single strain might have established itself in the second incubation mixture if the repeated sub-culture had been continued.

The faster-growth character was unstable in these variants (as seen previously) and the isolates reverted to a slow-growth phenotype (growth rate 85% of the wild-type) during 3 months storage at room temperature. Further, it would appear that the faster-growth character is not related to mt DNA as two isolates lacked all detectable mt DNA. To test this point, it was decided to conduct a 'faster-growth' experiment with strain 410, as *petite* mutants of this strain are known to lose their mitochondrial DNA very rapidly (see also Chapter 4).

(C.1) Faster-growing [*rho]* isolates from strain 410

Two well characterized spontaneous *petites* of 410 were chosen because each initially had a small amount of
mt DNA of unchanged buoyant density, (1.685 g/cm³).

It was found (Table 1-3C) that after 154 generations of continuous sub-culturing, the two petite strains were faster-growing with growth rates equal to that of the wild-type strain. Further, the suppressiveness of both petite strains was 1% when crossed to strain 4342, which correlated with the absence of any detectable mt DNA when a mitochondria-enriched fraction from the petite strains was centrifuged to equilibrium on a CsCl-EtBr density gradient and the upper region analysed (Fig. 1-11 c, e). Thus in the case of spontaneous petite isolates of strain 410, continuous culture leads to the appearance of faster-growing variants which lack all detectable mt DNA. The faster-growing character was also unstable in these last isolates, which once more reverted to a slow-growth phenotype.

Discussion

Three separate series of faster-growing [rho]⁻ strains have been isolated and characterised. The results presented in the previous Section (Section 1-1) demonstrated that all cytoplasmic petite strains grow at a slower rate than their corresponding wild-type strains in a glucose medium, a property of respiratory-deficient mutants unrelated to the presence or absence of intact mt DNA. In this section the results show that this slow-growth phenotype of petites is not an invariant character and that by repeated sub-culturing, petite strains with a faster doubling time can be isolated. However, not all faster-growing isolates have growth rates equal to that of the respiratory-competent parent.
FIG. 1-11

Uviscan traces of CsCl-buoyant density gradients in the analytical ultracentrifuge. The traces all correspond to linear top band fractions of mitochondrial-enriched particulate fractions prepared by identical procedures from: strain 410 [rho]+ (A) and its spontaneous petites 410 s[rho]⁻¹ (B) and 410 s[rho]⁻⁴ (D). The other two traces represent the same spontaneous petites after 154 generations continuous growth, when both petites were found to be faster-growing variants, i.e. 410 f[rho]⁻¹ (C) and 410 f[rho]⁻⁴ (E). DNA from Micrococcus luteus at a buoyant density of 1.731 g/cm³ is a marker.
Absorbance ($\lambda = 254$ n.m)

Wild-type

A

B

[rho]$^-$

C

after 154 gens

D

[rho]$^-$

E

after 154 gens

1.685 1.701 1.731 g/cm$^3$

Density g/cm$^3$ →
The technique used to isolate the faster-growing variants was basically the same in each case, involving repeated sub-culturing. However, in two of the situations the resultant incubation mixture varied. In the first case, a single faster-growing strain became dominant; whilst in the second case only 12% of the colonies present in the final incubation mixture were faster-growing variants, and at least three different strains were present in this faster-growing fraction. It is quite possible that the difference is due to the fact that this second incubation mixture has not reached the same stage of final selection as in the first repeated sub-culture.

In the third situation, individual petite isolates from strain 410 lost all detectable mt DNA on repeated sub-culture, and were found to be faster-growing variants. Thus in two separate situations it was found that faster-growing [rho]− isolates lacked all detectable mt DNA. Hence, the faster-growth character has no correlation with the presence or absence of mt DNA.

Nevertheless it is of interest to note that in the faster-growing [rho]− isolates that still retain mt DNA, this DNA was found to be of unchanged buoyant density and in the two cases analysed, was present as small circular molecules. In one isolate the mt DNA consists of an oligomeric series of circular DNA molecules having a unique monomeric size (1.5 μm), whilst in the other case, the [rho]− isolate possesses a heterogeneous pattern of circular DNA lengths not compatible with a single series of simple multiples. This situation could be due to the presence of two separate circle sizes of 0.5 and 0.8 μm. In both
isolates the mt DNA buoyant density is unchanged from that of the wild-type and the profile shows no heterogeneity. Examples of both homogeneous and heterogeneous sized circular mt DNA molecules have been reported in a series of EtBr-induced petite strains (Locker et al. 1974). In this study, a petite strain had mt DNA composed of three circular monomer sizes and of unchanged buoyant density, whilst the other petites, with only a single circular monomer size, had mt DNA of altered buoyant density if the circle monomer length was 1.2 μm or less. Hence the results of this study are in agreement with those published previously (Locker et al. 1974).

The o DNA profile reported previously in an EtBr-induced petite of strain 4545 (Clark-Walker 1973) was recognisable in the faster-growing [rho]− variants. This o DNA has two components, a DNA species of 2 μm and its oligomers, plus the other DNA species of 2.8 and 5.8 μm. An increase in the frequency of the 2.8 and 5.8 μm DNA species was found in a copper-resistant mutant from the EtBr-induced petite of strain 4545 (Clark-Walker and Miklos 1975B) and correlated with an increase in the heavy shoulder of the buoyant density traces. Due to its size and buoyant density, this other DNA species of 2.8 and 5.8 μm was postulated to be ribosomal DNA produced in the nucleus and rapidly exported (Clark-Walker and Miklos 1975B). Further, this DNA species is unstable (Clark-Walker, private communication) and, during storage on silica gel at 4°C, mutant strains lose the increased frequency of the 2.8 and 5.8 μm DNA species. This unstable character is directly analogous
to the situation observed with faster-growing variants in the present study.

Thus in the faster-growing [rho]− isolates which lacked mt DNA, special interest was taken to observe whether the faster-growth character correlated with an increase in the relative frequency of this 2.8 and 5.8 μm DNA component of o DNA. In the faster-growing isolates there is no detectable heterogeneity in the 1.701 g/cm³ peak of the buoyant density trace and the 2.8 and 5.8 μm DNA species only increased in frequency from the 10% of all circular molecules found in the EtBr-induced neutral petite to 13% of all circular DNA molecules. In the copper-resistant mutant the 2.8 and 5.8 μm DNA species accounted for 20% of all circular DNA molecules. Thus in the faster-growing [rho]− variants in the present study, there was no correlation observed between the faster-growth character and an increase in the relative frequency of the larger o DNA species. However, these results do not eliminate the possibility that there has been an increase in the total numbers of both species of closed-circular DNA relative to the normal situation.

In conclusion, the faster-growth character has no association with mt DNA and is unstable over a period of time. Unfortunately it does not at present shed any light on the slow-growth phenomenon.
Closed-Circular DNA from Mitochondrial-Enriched Fractions of Four *Petite*-Negative Yeasts

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Closed-circular DNA has been isolated from mitochondrial-enriched fractions from four ‘petite-negative’ yeasts. Electron microscope analysis has shown in each case the presence of a large discrete size class of circular DNA greater than 6 µm in length and smaller heterodisperse circular DNA less than 6 µm. Length and molecular weight measurements of the large circular DNA are: *Candida parapsilosis*, 11.14 ± 0.45 µm and 23.1 × 10^6; *Hansenula wingei*, 8.22 ± 0.43 µm and 17.3 × 10^6; *Kluyveromyces lactis*, 11.44 ± 0.20 µm and 24.0 × 10^6 and *Schizosaccharomyces pombe*, 6.04 ± 0.16 µm and 12.5 × 10^6. These circular DNAs are thought to be mitochondrial DNA on the basis of their buoyant densities and their enrichment with mitochondria.

The finding of a gross size difference between mitochondrial DNA from *petite*-negative yeasts and *Saccharomyces cerevisiae* focuses attention on the suggestion that certain unique properties of the latter organism’s mitochondrial DNA may be of relevance to the mechanism of the *petite* mutation in yeast.

**MATERIALS AND METHODS**

**Organisms**

Three haploid strains of sporogenous yeast and one asporogenous strain were used. *Hansenula wingei* V31B *met*^-^ and *Kluyveromyces lactis* W231B a, *his*^-^ were obtained from Dr A. I. Herman (Northern Regional Research Laboratory, Peoria, Illinois) and *Schizosaccharomyces pombe* was obtained from Dr E. H. Creaser (Department of Genetics, Research School of Biological Sciences, Australian National University). The asporogenous yeast, *Candida parapsilosis* was the same strain as used in a previous study [15].
Circular DNA in "Petite-Negative" Yeasts

The figure shows a supercoiled (C) and relaxed (A and B) molecules of the large-size class, together with smaller circular molecules (2.3, 2.2 and 1.0 μm, A). A circular λ molecule (14.6 μm, arrowed) is also illustrated. The figures are at a magnification of 30300 (A), 29700 (B) and 29600 (C). The bar in (C) represents 1 μm.

Fig. 1. Molecules from the closed-circular DNA band of C. parapsilosis. The figure shows a supercoiled (C) and relaxed (A and B) molecules of the large-size class, together with smaller circular molecules (2.3, 2.2 and 1.0 μm, A). A circular

Procedures

Culture conditions, fractionation of yeast, dye-buoyant density centrifugation, analytical ultracentrifugation and electron microscope visualization of DNA have all been described in detail previously [16,17]. Briefly, strains were grown overnight at 30 °C in 2% glucose—yeast—peptone medium containing 2% glucose by weight, 0.5% yeast extract, 1% bactopeptone and 0.3% KH₂PO₄. Cells were harvested, resuspended in sorbitol buffer, containing 0.5 M sorbitol, 0.05 M EDTA and 0.5 g ethidium bromide (EtBr)/l, and disrupted in a Braun homogeniser with glass beads. A mitochondrial-enriched pellet was obtained after three successive centrifugations to remove unbroken cells, wall debris and nuclei.

The mitochondrial-enriched pellet was resuspended in 2% sarkosyl, added to a CsCl-EtBr gradient and centrifuged to equilibrium in a Ti 50 rotor. The DNA band was visualised under ultraviolet light and extracted by side-puncture with an 18-gauge needle. The bound EtBr was removed with iso-amyl alcohol and the DNA dialysed overnight against 0.1 x standard saline citrate.

The buoyant density analyses were performed in a Spinco Model E ultracentrifuge under standard conditions, using Micrococcus luteus DNA as a marker.

For visualization, DNA was spread on a hypo-phase of 0.25 M NH₄Ac, picked up on parlodion films, stained in 0.05 mM uranyl acetate and observed at a magnification of 9800 in an Hitachi electron microscope. For molecular weight measurements the circular yeast DNA was spread with circularised λc1857 DNA, which was a generous gift of Dr A. Bellet (J.C.S.M.R., Australian National University). For calculations, the molecular weight of λ DNA was taken to be 30.8 x 10⁶ [18]. Lengths of relaxed circular molecules were measured with a map measurer, after projection of the negatives onto a screen giving an additional magnification of 17.3 times.

RESULTS

A closed-circular DNA band was seen in the preparative dye-buoyant density gradients of the mitochondrial-enriched fractions from all four 'petite-negative' yeasts. Examination in the electron microscope of the closed-circular DNA from the species revealed in each case the presence of two size classes of molecules (Fig.1—3). (The circular molecules from K. lactis have not been illustrated as they are of similar size to C. parapsilosis.) These two classes were characterised by a discrete class greater than 6 μm and small apparently heterodisperse molecules less than 6 μm in length (Fig.4—7). In K. lactis and S. pombe nearly all the circular molecules were large, but in C. parapsilosis and H. wingei the smaller molecules represented approximately 35—50% of the number present. Additionally, a considerable number of the small, circular molecules of H. wingei had 'tails' (Fig.2) These forms were also observed amongst the small molecules in the other three species but have not been included in any of the histograms.

For determination of the molecular weight of the circular molecules, each preparation was spread in the presence of circularised λ DNA (Fig.1 and 4—7), as in each case, no 15-μm size-class molecules were initially present. The molecular weights of the large-circular size class in each case are presented in Table 1.

Buoyant density determinations of both the closed-circular and linear DNA preparations are shown in Fig. 8 and the values obtained are presented in Table 2. In H. wingei, two species are present in the closed-circular DNA, having buoyant densities of 1.686 and 1.701 g/cm³, whereas in the other three strains only a single peak was observed. Moreover, in C. parapsilosis and S. pombe the density of the closed-circular DNA is similar to that of the main-band DNA, whereas the single peak in K. lactis and the major component in H. wingei are considerably lighter in density than the main-band DNA. These findings are supported by the observation of only a single linear DNA band in the preparative dye-buoyant density gradients from C. parapsilosis and S. pombe whereas
Fig. 3. *Molecules from the closed-circular DNA band of* S. pombe, *spread in the presence of ethidium bromide.* Two molecules of the large size-class, both 6.6 μm (A), and three smaller circular molecules, 2.2, 2.2 (B) and 0.3 (A) μm, are present. The figures are at a magnification of 29600. The bar in (A) represents 1 μm.

Fig. 4. *Length distribution of relaxed circular molecules from the closed-circular DNA band of* K. lactis *spread in the presence (B) and absence (A) of λ.* The number of molecules in the main peak is 55 (A) and 50 (B), and the number of molecules in the λ peak is 39 (hatched area).

Fig. 5. Length distribution of relaxed circular molecules from the closed-circular DNA band of *C. parapsilosis* spread in the presence (B) and absence (A) of λ. The number of molecules in the main peak is 33 (A) and 25 (B), and the number of molecules in the λ peak is 71 (hatched area). The number of small molecules account for 42% (A) and 53% (B) of the total.

Fig. 6. Length distribution of relaxed circular molecules from the closed-circular DNA band of *H. wingei* spread in the presence (B) and absence (A) of λ. The number of molecules in the main peak is 55 (A) and 98 (B), and the number of molecules in the λ peak is 37 (hatched area). The number of small molecules account for 40% (A) and 36% (B) of the total.

two upper, linear DNA bands are seen with *K. lactis* and *H. wingei*.

**DISCUSSION**

The observation of a major size class of circular DNA of length 11.4 μm in the mitochondrial-enriched fraction from *K. lactis* correlates with the presence of a single peak of buoyant density 1.692 g/cm³ in the analytical ultracentrifuge. These facts strongly support the contention that the 11.4-μm circles originate in the mitochondria. This view is also supported by reports that lighter density DNA satellites of fungi represent mitochondrial DNA [19] and other authors have ascribed a peak of density 1.692 g/cm³ in *K. lactis* to mitochondrial DNA [20,21]. By analogy the large-circle size class in the other three species can be ascribed to mitochondrial DNA, although in *C. para­psilosis* and *S. pombe* the buoyant densities do not differ significantly from main-band DNA. With *H. wingei*, where two buoyant density peaks are present in the analytical ultracentrifuge, the lighter peak almost certainly corresponds to the 8.2-μm circle size because of its sharper profile and greater amount. The small peak of buoyant density 1.701 g/cm³ in this preparation probably represents the density of some of the small circular DNA analogous to the situation in *S. cerevisiae* [16,17]. The problem of whether all the small circular DNA has the same buoyant density as main-band DNA is under investigation.

Guanine-plus-cytosine contents of the main-band DNA of all four species, calculated from the buoyant density data, are in the range of 37—42%. These values are similar to the G + C contents of DNA from these species determined by other methods [22—24].  

The circular mitochondrial DNA from the four ‘petite-negative’ yeasts is larger than most circular mitochondrial DNA from higher organisms [25—27] but is of similar size to that reported from Paramecium (12.8 μm) [28]. On the other hand the sizes are smaller than the circular mitochondrial DNA from other fungi such as Saprolegnia (14 μm) [29], Neurospora (19—20 μm) [30, 31] and two ‘petite-positive’ yeasts S. cerevisiae and S. carlsbergensis (21 — 25 μm) [3, 4].  

The present findings of the gross size difference in circular mitochondrial DNA between the ‘petite-positive’ and ‘petite-negative’ yeasts focuses attention on the suggestion that certain unique properties of S. cerevisiae mitochondrial DNA may be of relevance to the mechanism of the petite mutation in yeast [8].

We thank Dr W. J. Peacock for help with the DNA buoyant density analysis and Dr G. L. G. Miklos for many useful discussions.

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Note Added in Proof (March 3, 1975). The mitochondrial DNA of S. pombe has been reported to have a buoyant density of 1.689 g/cm³ [Bostock, C. J. (1969) Biochim. Biophys. Acta, 195, 579—581]. In the present study the circular DNA from S. pombe was found to have a buoyant density of 1.695 g/cm³. The question of whether the circular DNA is indeed of mitochondrial origin is presently under investigation.

CHAPTER 2

STUDIES ON CLOSED-CIRCULAR DNA ISOLATED FROM
MITOCHONDRIAL-ENRICHED FRACTIONS OF PETITE-NEGATIVE YEASTS

SECTION 2-1

ISOLATION AND CHARACTERIZATION OF CLOSED-CIRCULAR MITOCHONDRIAL DNA FROM FOUR PETITE-NEGATIVE YEASTS
Introduction

Petite-negative yeasts which account for over 70% of those tested (Bulder 1964A), fail to yield viable, cytoplasimically-inherited, respiratory-deficient mutants either spontaneously or when subjected to petite-inducing agents (c.f. Introduction of this thesis). Two possible explanations for the phenomenon of petite-negativity have been advanced, based on either the presence of viability genes on the mt DNA (c.f. Chapter 1) or the lack of certain structural features in their mt DNA (Clark-Walker and Miklos 1974B).

As an approach to testing these suggestions, the closed-circular DNA, containing the mitochondrial DNA, was isolated from mitochondria-enriched fractions from a number of petite-negative species. The yeasts chosen for study included three haploid ascomycetous species from which viable chromosomally-inherited, respiratory-deficient mutants have been isolated. These haploid species were Hansenula wingei (Crandall 1973B), Kluyveromyces lactis (Herman and Griffin 1968; Del Guidice and Puglisi 1974) and Schizosaccharomyces pombe (Wolf et al. 1971; Heslot et al. 1970B) A fourth, asporogenous species Candida parapsilosis was also included in the study as this yeast has been well characterized biochemically (Linnane et al. 1968A).

Materials and Methods

Organisms

The sources of the four yeast species are detailed in Appendix I.
**Methods**

Culture conditions, fractionation of yeast, dye-buoyant density centrifugation, analytical ultracentrifugation and electron microscope visualization of DNA, are all described in detail in Appendix I.

Briefly, growth analysis were performed in a New Brunswick water-bath at 30°C and 200 rpm in a 2% glucose-yeast peptone medium (GYP) containing 2% glucose by weight, 0.5% yeast extract, 1% bactopeptone and 0.3% potassium dihydrogen orthophosphate (KH$_2$PO$_4$).

For DNA preparation, the cultures were grown overnight at 30°C in the same 2% GYP medium and harvested at a specific point on the growth cycle as detailed below. The cells were resuspended in a sorbitol buffer (ESE) containing 0.5M sorbitol, 0.05M EDTA and 0.5 grams/litre ethidium bromide (EtBr) and disrupted in a Braun homogeniser with glass beads. A mitochondrial-enriched pellet was obtained after three successive centrifugations to remove unbroken cells, wall debris and nuclei. This mitochondrial-enriched pellet was resuspended in 2% sarkosyl, added to a caesium chloride-ethidium bromide-EDTA gradient and centrifuged to equilibrium in a Beckman 50Ti rotor. The DNA bands were visualized by ultraviolet light excited fluorescence of the intercalated EtBr ($\lambda = 350$ nm) and removed by side-puncture of the tube using an 18-gauge needle. The bound EtBr was removed with iso-amyl alcohol and the DNA dialysed overnight against 0.1 x standard saline citrate (SSC).

The buoyant density analyses were performed in a
Spinco Model E or an MSE Centriscan analytical ultracentrifuge under standard conditions, using main band DNA from *Micrococcus luteus* as a marker.

For visualization, DNA was spread on a hypophase of 0.25M ammonium acetate, picked up on parlodion films, stained in 0.05 mM uranyl acetate and observed at a magnification of around 10,000 in an Hitachi HUIIE or a Jeol 100C electron microscope. For molecular weight determinations the circular yeast DNA was spread with circularized λcI 857 DNA which was a generous gift of Dr. A. Bellet (J.C.S.M.R., Australian National University). For calculations, the molecular weight of λ DNA was taken to be 30.8 x 10^6 daltons (Davidson and Szybalski 1971). Lengths of relaxed circular molecules were measured with a map measurer after projection of the electron micrograph negatives onto a screen giving an additional magnification of 17.3 times.

**Results**

A closed-circular DNA band was observed in the preparative dye-buoyant density gradients of the mitochondrial-enriched fractions from the four petite-negative yeasts. An extensive study with *C. parapsilosis* showed that the point on the growth cycle at which the cells were harvested is important for the appearance of this closed-circular DNA band. The best yield of closed-circular DNA was obtained when the culture was harvested in the late exponential/early stationary phase of the growth cycle. Hence, growth analyses were performed on the yeasts used.
A. Growth analysis of petite-negative yeasts

Growth characteristics of the yeasts are summarized in Table 2-1 and the growth curves illustrated (Fig. 2-1). The point at which the cultures were harvested to obtain the closed-circular DNA band is indicated by the arrow (Fig. 2-1).

The strains vary considerably in their growth rates but in all cases the cultures were harvested in late logarithmic or early stationary phase, when the culture density was between 2.5-4.5 mgs dry weight of cells/ml.

B. Dye-buoyant density gradients

When mitochondrial-enriched pellets from the petite-negative yeast species are centrifuged to equilibrium on a CsCl-EtBr gradient, two upper linear DNA bands and a lower, closed-circular DNA band are visible with K. lactis and H. wingei (photo 2-1), whilst only a single upper band and a lower, closed-circular band are seen with C. parapsilosis and S. pombe.

For each petite-negative yeast, two DNA fractions are removed by side puncture of the polyallomer tube. The first fraction, termed 'the top DNA band' (Fraction A, photo 2-1), comprises the uppermost band of the linear DNA part of the gradient, together with some of the lower, main band DNA. The second fraction, termed 'the closed-circular DNA band' (Fraction B, photo 2-1), comprises the lower closed-circular DNA band visible in the CsCl-EtBr gradients of the petite-negative yeasts.

C. Buoyant density analysis of both DNA fractions

After EtBr extraction and dialysis, both the 'top
## TABLE 2-1

Growth data of *petite*-negative yeasts in a 2% GYP medium at 30°C

<table>
<thead>
<tr>
<th>Organism</th>
<th>Doubling time (mins)</th>
<th>Relationship of dry wt. to optical density, i.e. an OD&lt;sub&gt;640&lt;/sub&gt; nm of 0.1 is given by a dry wt of:</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. parapsilosis</em></td>
<td>64</td>
<td>0.018 mgs dry wt/ml</td>
</tr>
<tr>
<td><em>H. wingei</em></td>
<td>83</td>
<td>0.020</td>
</tr>
<tr>
<td><em>K. lactis</em></td>
<td>87</td>
<td>0.018</td>
</tr>
<tr>
<td><em>S. rosei</em></td>
<td>97</td>
<td>0.023</td>
</tr>
<tr>
<td><em>S. pombe</em></td>
<td>123</td>
<td>0.018</td>
</tr>
<tr>
<td><em>S. rouxii</em></td>
<td>180</td>
<td>0.030</td>
</tr>
</tbody>
</table>
FIG. 2-1

Growth analysis of petite-negative yeasts. The figure illustrates the growth to stationary phase of (A) *C. parapsilosis*, (B) *K. lactis*, (C) *H. wingei* in a 2% GYP medium at 30°C. The culture density was determined by measuring the optical density at 640 nm of a 1 ml aliquot at various times and plotted as log_{10} O.D. 640 nm vs. time (hours). The arrow in each figure shows the point of harvest for a DNA preparation at the late exponential/early stationary phase of the growth cycle.
A caesium-chloride, ethidium-bromide buoyant density gradient of a mitochondria-enriched fraction from *H. wingei*. The photograph is taken through an orange-UV filter using $\lambda = 350$ nm. Both the top-band DNA fraction (A) and the lower, closed-circular DNA band fraction (B) are indicated. The fractions are removed by side-puncture of the polyallomer tubes and withdrawn with a 1 ml syringe.
DNA band' and the 'closed-circular DNA band' were analysed by analytical ultracentrifugation. For buoyant determinations of the components in each fraction, *Micrococcus luteus* DNA was added as a marker. The results are illustrated in Fig. 2-2, and summarized in Table 2-2.

*K. lactis* had two components of buoyant densities 1.700 and 1.692 g/cm³ in its 'top DNA band' but only a single component of buoyant density 1.692 g/cm³ in its closed-circular DNA band (Fig. 2-2 A, B). Similarly, *H. wingei* had components of buoyant density 1.701 and 1.686 g/cm³ in its upper bands and the same two components were present in the 'closed-circular DNA band' (Fig. 2-2 G, H). *C. parapsilosis* and *S. pombe* each had a single component of buoyant density 1.700 and 1.696 g/cm³ respectively in their upper linear DNA band and a single component of buoyant density 1.658 and 1.695 g/cm³ respectively in their closed-circular DNA band (Fig. 2-2 C, D and E, F).

**D. Electron microscope analysis of the closed-circular DNA bands**

The 'closed-circular DNA band' from each petite-negative yeast was analysed by electron microscopy. In each case, the analysis revealed the presence of two distinct size classes of DNA molecules, characterized in the first case by a discrete size class of molecules greater than 6 μm in length and secondly, by a class of small, apparently heterodisperse molecules less than 6 μm in length.

For the molecular weight determination of the large size class of DNA molecules, a separate preparation of the
Uviscan traces of CsCl-buoyant density gradients in the analytical ultracentrifuge. The traces correspond to linear DNA bands (A, C, E, G) and the closed-circular DNA band (B, D, F, H) from mitochondrial-enriched fractions of respectively: (A, B) *K. lactis*, (C, D) *C. parapsilosis*, (E, F) *S. pombe*, (G, H) *H. wingei*. DNA from *Micrococcus luteus* at a buoyant density of 1.731 g/cm³ is a marker.
**TABLE 2-2**

Buoyant densities of linear and closed-circular DNA from *petite*-negative yeasts

<table>
<thead>
<tr>
<th>Organism</th>
<th>Buoyant density (g/cm³) of:</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Linear DNA</td>
<td>Closed circular DNA</td>
<td>Main band DNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K. lactis</td>
<td>1.7004, 1.6921</td>
<td>1.6915</td>
<td>1.7004</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. parapsilosis</td>
<td>1.7000</td>
<td>1.6983</td>
<td>1.7000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H. wingei</td>
<td>1.7012, 1.6863</td>
<td>1.7016, 1.6864</td>
<td>1.7012</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. pombe</td>
<td>1.6969</td>
<td>1.6953</td>
<td>1.6969</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. rouxii</td>
<td>1.7018, 1.6922</td>
<td>1.7023</td>
<td>1.7018</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. rosei</td>
<td>1.7014, 1.6934</td>
<td>-</td>
<td>1.7014</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

These figures represent the average of at least two buoyant density determinations of each fraction.
'closed-circular DNA band' was spread in the presence of circularized λ DNA, as in each case no 15 μm class of DNA molecules was initially present in any of the fractions. The circularized λ DNA (Fig. 2-3) had a mean length 14.61 μm and only one smaller molecule, 0.4 μm in length (Fig. 2-3, 2-4) was observed.

The 'closed-circular DNA band' from *K. lactis* contains a major size class of large DNA molecules 11.44 μm in length (Fig. 2-5, 2-6) of molecular weight 24.0 x 10^6 daltons (Table 2-3), with only a few, smaller, heterodisperse molecules. Similarly, *C. parapsilosis* had a large size class of DNA molecules 11.14 μm long (Fig. 2-7, 2-8) and molecular weight 23.1 x 10^6 daltons (Table 2-3). The smaller, heterodisperse molecules present in the 'closed-circular DNA band' of *C. parapsilosis* account for between 40-50% of the total number of relaxed molecules viewed.

The 'closed-circular DNA band' from *H. wingei* has a large size class of DNA molecules 8.22 μm in length (Figs. 2-9, 2-10) of molecular weight 17.3 x 10^6 daltons (Table 2-3). The smaller DNA molecules again accounted for about 40% of the total relaxed molecules and apparently contained different size classes (c.f. Section 2-3 of this thesis). Additionally, a considerable number of the small circular DNA molecules of *H. wingei* had 'tails' (Fig. 2-9). These forms were also observed at a lower frequency amongst the smaller molecules in the other three species but have not been included in any of the histograms.

Finally, the 'closed-circular DNA band' of *S. pombe* contained the smallest, discrete size class of DNA molecules 6.04 μm in length (Figs. 2-11, 2-12) and molecular weight
Circular DNA molecules from lambda strain λcI 857, reannealed by gradual cooling from 70°C, used as a standard in molecular weight determinations. The figure shows a molecule of length 14.7 μm (A) plus the only small molecule seen in the preparation of length 0.4 μm (B). The figures are at a magnification of 29,900. The bar in (A) represents 1 μm.
Length distribution of relaxed circular DNA molecules from λcI 857 reannealed by gradual cooling from 70°C. The number of molecules in the main peak is 49, with a mean length of 14.61 ± 0.46 μm. Some larger molecules of dimer size are also present. Only one smaller DNA molecule (length 0.4 μm) was seen.
Molecules from the closed-circular DNA band of *K. lactis*. The figure shows a relaxed (length 11.6 μm) and a more supercoiled molecule of the large size class. The figure is at a magnification of 30,000. The bar represents 1 μm.
FIG. 2-6

Length distribution of relaxed circular molecules from the closed-circular DNA band of *K. lactis* spread in the presence of (B) and absence (A) of λ. The number of molecules in the main peak is 55 (A) and 50 (B) and the number of molecules in the λ peak is 39 (hatched area).
FIG. 2-7

Molecules from the closed-circular DNA band of C. parapsilosis. The figure shows a supercoiled (C) and relaxed (A and B) molecules of the large size class, together with smaller circular molecules (2.3, 2.2 and 1.0 μm, A). A circular λ molecule (14.6 μm, arrowed) is also illustrated. The figures are at a magnification of 30,000 (A), 29,700 (B) and 29,600 (C). The bar in (C) represents 1 μm.
FIG. 2-8

Length distribution of relaxed circular molecules from the closed-circular DNA band of *C. parapsilosis* spread in the presence (B) and absence (A) of λ. The number of molecules in the main peak is 33 (A) and 25 (B), and the number of molecules in the λ peak is 71 (hatched area). The number of small molecules account for 42% (A) and 53% (B) of the total.
FIG. 2-9

Molecules from the closed-circular DNA band of *H. wingei*. The figure shows a molecule of the large size class (A) with two smaller circular molecules, 3.8 (B) and 1.0 (A) μm. Also present are three molecules from the small-size class that have a 'tail', 2.8 (B), 2.2 (C) and 0.6 (A) μm, or a bubble, 3.2 (D) μm; these types of molecules have not been included in the histograms. The figures are at a magnification of 28800 (A and D) and 28900 (B and C). The bar in (A) represents 1 μm.
FIG. 2-10

Length distribution of relaxed circular molecules from the closed-circular DNA band of *H. wingei* spread in the presence (B) and absence (A) of λ. The number of molecules in the main peak is 55 (A) and 98 (B), and the number of molecules in the λ peak is 37 (hatched area). The number of small molecules account for 40% (A) and 36% (B) of the total.
Length distribution of relaxed circular molecules from the closed-circular DNA band of *S. pombe* spread in the presence (B) and absence (A) of λ. The number of molecules in the main peak is 79 (A) and 38 (B), and the number of molecules in the λ peak is 36 (hatched area).
TABLE 2-3

Length and molecular weight data of the mitochondrial DNA from four petite-negative yeasts

<table>
<thead>
<tr>
<th>Organism</th>
<th>Length of mt DNA (µm)</th>
<th>Length of λ DNA (µm)</th>
<th>Mol. wt. of mt DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>K. lactis</em></td>
<td>11.44 ± 0.20</td>
<td>14.67 ± 0.22</td>
<td>24.0 x 10^6</td>
</tr>
<tr>
<td><em>C. parapsilosis</em></td>
<td>11.14 ± 0.45</td>
<td>14.85 ± 0.51</td>
<td>23.1 x 10^6</td>
</tr>
<tr>
<td><em>H. wingei</em></td>
<td>8.22 ± 0.43</td>
<td>14.57 ± 0.38</td>
<td>17.3 x 10^6</td>
</tr>
<tr>
<td><em>S. pombe</em></td>
<td>6.04 ± 0.16</td>
<td>14.92 ± 0.32</td>
<td>12.5 x 10^6</td>
</tr>
</tbody>
</table>
12.5 x 10^6 daltons (Table 2-3). Very few smaller circular DNA molecules were present in this species.

Discussion

A closed-circular DNA band can be isolated from mitochondrial-enriched fractions in CsCl-EtBr buoyant density gradients, due to differential binding of EtBr by three DNA species (closed-circular, single-stranded nicked and double-stranded nicked or linear) (Radloff et al. 1967; Hudson et al. 1969; Bauer and Vinograd 1968). Dye-binding to DNA reduces the buoyant density of the complex and at saturating levels of EtBr, closed-circular DNA binds less dye than open circular or linear DNA due to an unwinding constraint.

The observation of a major size class of circular DNA molecules of length 11.4 μm in the 'closed-circular DNA band' from the mitochondrial-enriched fraction of K. lactis correlates with the presence of a single peak of buoyant density 1.692 g/cm^3 in the analytical ultracentrifuge. These facts strongly support the contention that the 11.4 μm circles originate in the mitochondria. Further support for this contention comes firstly from a report that lighter density DNA satellites of fungi represent mt DNA (Villa and Storck 1968) and secondly, from other authors who have ascribed a peak of density 1.692 g/cm^3 in K. lactis to mt DNA (Smith et al. 1968; Luha et al. 1971). Additionally, Sanders et al. (1974) report similar DNA molecules of length 11.4 μm in a mitochondrial lysate of K. lactis and ascribe the circles to mt DNA. The linear mt DNA isolated in their preparation had a kinetic complexity of 20 x 10^6 daltons.
Smith et al. (1968) using a Cs₂SO₄-Hg²⁺ analytical gradient to separate the two DNA species in a whole cell DNA preparation of *K. lactis*, find that mt DNA represents 14% by mass of the total cell DNA. Assuming that the haploid genome size of *K. lactis* is the same as that of *S. cerevisiae* (5000 μm) (Hartwell 1970), then a haploid cell of *K. lactis* contains a total length of 700 μm of mt DNA. As the mt genome size is 11.4 μm, this total mt DNA represents 61 copies of the mt genome per haploid cell in *K. lactis*, a value similar to that reported for *S. cerevisiae* (Nagley and Linnane 1970; Williamson 1970).

By analogy with *K. lactis*, the large size class of DNA molecules in the other three species can be ascribed to mt DNA, although in *C. parapsilosis* and *S. pombe*, the buoyant densities do not differ significantly from those of the main band DNA.

With the 'closed-circular DNA band' of *U. wingei* where two buoyant density peaks are present in the analytical ultracentrifuge, the lighter peak almost certainly corresponds to the 8.2 μm DNA circles because of its sharper profile and greater amount. The smaller peak of buoyant density 1.701 g/cm³ in this preparation probably represents the density of some of the smaller circular DNA, analogous to the situation in *S. cerevisiae* (Clark-Walker 1972, 1973) and *S. rouxii* (Section 2-2 of this thesis). The problem of whether all the smaller circular DNA has the same buoyant density as main band DNA, is discussed later (Section 2-3 of this thesis).
The circular mt DNA from the four petite-negative yeasts is larger than most circular mt DNA from higher organisms (Borst 1972; Borst and Kroon 1969; van Bruggen et al. 1968); but that from *K. lactis* and *C. parapsilosis* is of similar size to those reported from the protozoan *Acanthamoeba castellanii* (12.8 µm) (Bohnert 1973; Bohnert and Herrman 1974), the avian malarial parasite *Plasmodium lophurae* (10.3 µm) (Kilejian 1975) and the ascomycete fungus, *Aspergillus nidulans* (10.5 µm) (Lopez Perez and Turner 1975). On the other hand, all of the sizes are smaller than the circular mt DNA from other fungi such as *Saprolegnia* (14 µm) (Clark-Walker and Gleason 1973), *Neurospora* (19-20 µm) (Clayton and Brambl 1972; Agsteribbe et al. 1972) and two petite-positive yeasts *S. cerevisiae* and *S. carlsbergensis* (21-25 µm) (Hollenberg et al. 1970; Petes et al. 1973). Pea leaf mitochondria also have a larger circular mt DNA of 30 µm (Kolodner and Tewarii 1972).

Analytical ultracentrifugation studies showed that the guanine-plus-cytosine contents of the main-band DNA of all four species, calculated from the buoyant density data, are in the range of 37-42%. These values are similar to the G + C contents of DNA from these species determined by other methods (Stenderup and Bak 1968; Storck 1966; Nakase and Komagata 1969). In the case of *S. pombe*, the buoyant density results reported here for the main band DNA agree with a previous report (Bostock 1968) whilst those reported for the mt DNA do not. In Bostock's communication, he reports the mt DNA to be a slight shoulder on the main-band peak in whole cell DNA extracts similar to the present observations using mitochondrial-enriched fractions (Fig.
2-2), however, Bostock further reports that his purified mt DNA has a buoyant density of 1.698 g/cm$^3$. This value does not agree with the 1.695 g/cm$^3$ buoyant density of the purified 6 μm circular DNA found here.

The buoyant densities of the mt DNA of three of the four petite-negative species, *S. pombe*, *C. parapsilosis* and *K. lactis*, were much higher (greater mole percent G + C) than the buoyant density of *S. cerevisiae* mt DNA (1.684 g/cm$^3$). In view of the fact that the low buoyant density of *S. cerevisiae* mt DNA is associated with AT-rich sequences (Piperno *et al.* 1972; Prunell and Bernardi 1974), it is possible that these three petite-negative yeasts lack these AT-rich sequences, and hence are unable to undergo excision-insertion events to produce defective genomes.

In the case of *H. wingei*, although the mt DNA is more AT-rich (buoyant density 1.686 g/cm$^3$) it is possible that both this mt DNA and other AT-rich mt DNAs from petite-negative yeasts (Bak *et al.* 1969), may still lack the regions of homology necessary for the excision-insertion events.
SECTION 2-2

CLOSED-CIRCULAR DNA FROM A PETITE-NEGATIVE YEAST

SACCHAROMYCES ROUXII
Introduction

In the previous section, it has been demonstrated that there exists a gross size difference between the reported size of mt DNA of the petite-positive yeasts *S. cerevisiae* and *S. carlsbergensis* and the mt DNA of four petite-negative yeasts outside this genus. It was then of interest to determine whether the mt DNA of petite-negative *Saccharomyces* species is also different in size from that of *S. cerevisiae*.

The genus *Saccharomyces* is phylogenetically heterogeneous and contains both petite-positive and petite-negative species. A recent study has detailed four recognisable groups comprising the genus "Saccharomyces sensu lato" (van der Walt 1970A).

The first of these groups includes species closely related to the type species *S. cerevisiae* and termed "Saccharomyces sensu stricto". Of the 17 species in this group, six species have been tested with petite-inducing agents and all six species are found to be petite-positive (Bulder 1964A, and Appendix II of this thesis). The second group consists of species closely related to *S. bailii*. The four species comprising this group, which includes *S. rouxii*, have all been found to be petite-negative. The third group comprises species closely related to *S. rosei*. Five species, out of the ten species included in the group, have been found to be petite-negative, whilst a sixth species, *S. kloeckerianus*, whose position in the group is disputed, has been found to be petite-positive. The fourth group is itself phylogenetically heterogeneous, comprising species for which there is little or no evidence of any phylogenetic
connection or relationship. The group consists of 10 species and of the five species tested, four have been found to be petite-positive and the fifth petite-negative.

The composition of these groups is discussed more fully in Appendix II of this thesis. In general terms, it appears that the first group, "Saccharomyces sensu stricto", consists of petite-positive species, whilst the second and third groups consist of petite-negative species. The fourth group is a mixed collection of species.

Thus the petite-negative, Saccharomyces species chosen for examination were S. rouxii and S. rosei, being representative of the second and third groups, respectively (Appendix II).

Materials and Methods

Organisms

Saccharomyces rouxii (C.B.S. 732) and Saccharomyces rosei (C.B.S. 817) were obtained from the Centraalbureau voor Schommelcultures (C.B.S.), Yeast Division, Delft, The Netherlands. Single colony isolates of both strains were used in this work.

Methods

Growth analyses, DNA preparation and analysis were the same as outlined earlier and detailed in Appendix I, with the exception of the phenol extraction to remove DNA-associated protein, detailed under "Preparation of DNA from Micrococcus luteus" in Appendix 1.

Results

A. Growth analyses

Both strains were found to grow slower than strains
of *S. cerevisiae* at 30°C with *S. rouxii* having a doubling time twice that of *S. rosei* (Table 2-1).

### B. Dye-buoyant density gradients

As with the other petite-negative strains, the *Saccharomyces* cultures were harvested in the late exponential/early stationary phase of growth and a mitochondrial-enriched pellet added to an EtBr-CsCl gradient. After centrifugation and visualization of the gradients under UV light, both species were observed to contain two upper, linear DNA bands, whilst only *S. rouxii* was seen to have a lower, closed-circular DNA band. The two fractions described previously, the 'top DNA band' and the lower 'closed-circular DNA band', were removed by side puncture of the centrifuge tubes.

### C. Buoyant density analysis of both DNA fractions

Analysis of the linear, top DNA bands using *M. luteus* DNA as a marker, showed the presence of main band DNA of buoyant density 1.701 g/cm³ in both species, similar to that of *S. cerevisiae*. The lighter DNA peaks had densities of 1.692 g/cm³ for *S. rouxii* and 1.693 g/cm³ for *S. rosei* (Fig. 2-13, Table 2-2).

The closed-circular, DNA band from *S. rouxii* contained a single DNA species of buoyant density 1.701 g/cm³ (Fig. 2-13B), similar to that of its main band DNA. No DNA was detected by analytical ultracentrifugation when the closed-circular DNA region from the *S. rosei* gradient was analysed.
Uviscan traces of CsCl-buoyant density gradients in the analytical ultracentrifuge of DNA isolates from petite-negative yeasts of the genus Saccharomyces. The traces correspond to linear DNA bands (A, C) and the closed-circular DNA band (B) from respectively: (A, B) S. rouxii and (C) S. rosei. The S. rosei mt DNA shows no sign of any main-band DNA contamination. DNA from Micrococcus luteus at a buoyant density of 1.731 g/cm³ is a marker.
D. Electron microscope analysis of the closed-circular DNA band

Preliminary work on the closed-circular DNA band from *S. rouxii*, showed that the DNA still had a considerable amount of protein associated with it, making the DNA very hard to visualize. The protein was removed by phenol treatment of the DNA as detailed in Appendix I.

Electron microscope analysis of the protein-free, closed-circular DNA, revealed the presence of a size class of DNA molecules about 1.8 μm in length, together with oligomeric forms of this monomer size class (Fig. 2-14, 2-15). No other covalently-closed, circular DNA was observed.

Discussion

The results of this Section show that both *S. rouxii* and *S. rosei* have mt DNA of higher buoyant density than *S. cerevisiae*. The buoyant density of the main band DNA of both *Saccharomyces petite*-negative species is similar to that of *S. cerevisiae*. It was impossible to isolate any closed-circular mt DNA from either strain and the closed-circular DNA band from *S. rouxii* contained a single DNA species of main-band buoyant density. Hence, it is not possible to determine the size of the mt DNA of these *petite*-negative species by electron microscopy and one would have to turn to the technique of renaturation kinetics (c.f. Chapter 3). Until this is done, one cannot answer the question as to whether a gross size difference exists between the mt DNA of *petite*-positive and *petite*-negative...
Molecules from the closed-circular DNA band of *S. rouxii* spread after phenol extraction of bound protein. The figure shows both relaxed and supercoiled molecules of monomeric length (1.8 μm) plus relaxed and supercoiled oligomeric sized molecules. The figure is at a magnification of 29,200. The bar represents 1 μm.
FIG. 2-15

Length distribution of relaxed circular molecules from the closed-circular band of *S. rouxii*. The number of molecules in the main peak is 85 with a mean length of $1.8 \pm 0.06 \, \mu m$, in the second peak is 12 (mean length $3.54 \pm 0.12 \, \mu m$) and in the third peak is 3 (length $5.3 \pm 0.1 \, \mu m$).
yeasts of the same genus.

The inability to isolate any closed-circular mt DNA from either *Saccharomyces* strain studied, is a situation similar to that found with *S. cerevisiae* but different from the other petite-negative yeasts studied. The reasons for this might be two-fold, either the conditions under which the mitochondrial-enriched fraction is isolated are incorrect, or else the endogenous nucleases of the cell might be more active in the *Saccharomyces* species than in other species. The latter reason seems the more valid, as harvesting *S. rosei* under different conditions of growth, from early exponential to late stationary, fails to yield any closed-circular DNA, let alone closed-circular mt DNA. With *S. rouxii* a closed-circular DNA band can be isolated, but this contains no closed-circular mt DNA, only a DNA species resembling closely omicron DNA of *S. cerevisiae* (c.f. below). Further, a recent report indicates that mitochondria of *S. cerevisiae* contain an endonuclease that degrades mt DNA preferentially at A + T-rich sites both *in vitro* and *in vivo* (Zeman and Lusena 1975). Thus it appears that the cell nucleases of the genus *Saccharomyces* do not permit the isolation of intact, closed-circular mt DNA.

The homogeneous size class of DNA molecules isolated from the closed-circular DNA band of *S. rouxii*, of monomer size length 1.8 μm and buoyant density similar to that of main-band DNA, 1.702 g/cm³, resemble closely omicron DNA found in the same closed-circular DNA band of *S. cerevisiae* (Clark-Walker 1972). Omicron DNA has a size length of 1.9 μm and a buoyant density similar to main-band DNA.
1.701 g/cm³. Unlike *S. cerevisiae*, the omicron DNA isolated as a single DNA species from *S. rouxii* shows no sign of a second, minor DNA species, postulated to be ribosomal DNA (c.f. Section 1-2 of this thesis), of length 2.85 μm, isolated along with omicron DNA in the closed-circular DNA band of a neutral petite strain of *S. cerevisiae* (Clark-Walker 1973). This study reveals that there is no relationship between omicron DNA and the response of a species to petite-inducing agents, as is shown by the detection of omicron DNA in a petite-negative species of *Saccharomyces*, a fact that will be discussed in the next Section dealing with the closed-circular DNA band of *H. wingei*.

The presence of small, closed-circular DNA molecules in *S. cerevisiae* has been described through the use of electron microscopy by Sinclair *et al.* (1967), where the DNA had a mean length of 1.95 μm, by Avers *et al.* (1968), Billheimer and Avers (1969), lengths around 2 to 2.5 μm and 5.6 to 6 μm, by Hollenberg *et al.* (1970), length around 2 μm, and has been isolated as a discrete DNA species by Guerineau *et al.* (1971), length around 2.2 μm and Clark-Walker (1972), length around 1.9 μm. The latter two studies showed that this class of DNA molecules had the same buoyant density as main-band DNA and was present in both [rho]⁺ and [rho]⁰ cells, which contain, respectively, intact mt DNA and no detectable mt DNA in the cell.

To date, no function has been ascribed to omicron DNA. Early postulates that this DNA species represents the gamma (γ) satellite found in association with the yeast
nuclear DNA or that it represents mt DNA, have been dis-
counted on the basis of buoyant density studies. \( q \) DNA
does not occur in purified yeast nuclei (Clark-Walker and
Miklos, 1974) and so cannot represent a gene amplification
product or be related to informosomal DNA (Bell 1969;
Chepelinsky and Bell 1972; Bell et al. 1972), since both
hypotheses require the presence of at least some \( q \) DNA in
the nucleus.

Recently a suggestion has been forwarded that
\( q \) DNA represents a DNA species necessary for mitochondrial
function and which has the properties of a plasmid or an
episomal system associated with mt DNA. This suggestion
stems, firstly, from the demonstration that cytoplasmic
triethyl-tin resistance and a class of cytoplasmic venturi-
cidin resistance determinants are unlinked to other mitochon-
drial loci (Griffiths et al. 1975) and, secondly, that
multiresistance determinants for oligomycin, venturicidin,
triethyl-tin, cycloheximide and chloramphenicol appear to
be nuclear mutations, but possess episomal characteristics
such as loss during vegetative multiplication and elimin-
ation during growth in ethidium bromide (Guerineau et al.
1974). The latter study demonstrated a correlation between
loss of the oligomycin resistance determinant and loss of
detectable \( q \) DNA. Further to this idea for the function
of \( q \) DNA, there exists a non-Mendelian mutation (URE3) in
S. cerevisiae which changes many of the enzymatic activities
linked to nitrogen metabolism. Early work has shown that
this determinant is not located on mt DNA (Lacroute 1971)
and recently, cytoduction experiments have shown that the
inheritance of the determinant is not linked to the nucleus,
but enters the receptor strain, which is *petite*, at the same time as the [rho]$^+$ mitochondria (Aigle and Lacroute 1975). A possible location on o DNA has been suggested for this determinant (Clark-Walker 1973).

In this Section it has been demonstrated that there is no relationship between o DNA and the phenomenon of *petite*-negative yeasts. o DNA occurs in a *Saccharomyces* petite-negative species and may occur in other *petite*-negative species of other yeast genera.
SECTION 2-3

ANALYSIS OF THE SMALL, CLOSED-CIRCULAR DNA MOLECULES ISOLATED FROM THE MITOCHONDRIAL-ENRICHED FRACTION OF THE PETITE-NEGATIVE YEAST HANSENULA WINGEI
Introduction

In the first Section of this chapter, it has been shown that small, heterodisperse DNA molecules can account for up to half of the total number of relaxed DNA molecules seen in the closed-circular DNA band of two petite-negative yeasts, H. wingei and C. parapsilosis. Furthermore, a number of these small DNA molecules are observed to possess 'tails' or 'bubbles' reminiscent of replicating intermediates reported in other mt DNAs (Arnberg et al. 1971, 1973; Kasamatsu et al. 1971; Robberson and Clayton 1971).

Some, perhaps all of these small circular DNA molecules in H. wingei must account for the diffuse peak of main band buoyant density found in the closed-circular DNA preparation. However, it was important to establish if any of these smaller, circular DNA molecules represented excision products from the mt DNA of this yeast. If this proved to be so, it would imply that the H. wingei mt DNA was capable of undergoing excision reactions even though these fragments were not able to exist in the absence of the complete genome.

In attempting to answer this question, the closed-circular DNA band of H. wingei has been analysed extensively. The results of this analysis show that the simple process of purifying the mitochondria on a sucrose gradient leads to the isolation of a closed-circular DNA band containing a single DNA species of mt DNA buoyant density and containing no small, circular DNA molecules. The question then arose as to the nature of these small DNA circles.
This has been studied by seeing if there are any discrete size classes amongst the small DNA circles both with and without 'tails'.

Materials and Methods

The *H. wingei* strain and the methods for preparing and analysing the closed-circular DNA band have been described (Section 2-1; Appendix I).

Results

A. Detailed analysis of the closed-circular DNA band of a crude mitochondrial-enriched fraction

A closed circular DNA band from a crude mitochondrial-enriched fraction of *H. wingei* was analysed by both analytical ultracentrifugation and electron microscopy.

As previously reported, both the lighter and heavier DNA species were seen to be present as shown by analytical ultracentrifugation (Fig. 2-16a). Accordingly the area under each curve was determined by use of a Muraki Compensating Planimeter. The lighter buoyant density species accounted for 54% of the DNA sample, whilst the heavier buoyant density species accounted for slightly less, 46%. Further, the sharper buoyant density profile of the lighter peak implies that the lighter peak represents a DNA species of higher molecular weight than that represented by the low, broad profile of the heavier buoyant density peak.

The electron microscope analysis showed the presence of a small amount of linear DNA, 14% of the total length of DNA viewed, practically all of which was under 4 μm in length. As previously described, the closed-circular DNA molecules consisted of two species, the large, discrete, size class
FIG. 2-16

Analysis of the closed-circular DNA band isolated from a crude mitochondrial-enriched fraction from *H. wingei*. The analysis is by both analytical ultracentrifugation (A) and electron microscopy (B, C).

In A, the areas under the peaks are 54% for the lighter and 46% for the heavier component as determined using a planimeter. *M. luteus* DNA of buoyant density 1.731 g/cm³ is used as marker.

B and C are histograms of the length distribution of the circular DNA molecules seen in the electron microscope. B represents the relaxed, small circular DNA with double-stranded 'tails' attached, whilst C represents the relaxed, open-circular DNA molecules seen. The number of molecules in the large-size class is 69 (C), average length 8.52 ± .38 μm, and in the small circular DNA region is 29 (C) and 65 (B). On a length basis, the large size class of DNA molecules and its oligomers account for 68% of the total length of DNA viewed, whilst the smaller molecules in both B and C account for 18% of the total length. A small proportion of linear DNA was seen, 14%, all of which was less than 4 μm in length.
of length 8.5 µm and the smaller, heterodisperse DNA molecules (Fig. 2-16c) the majority of which possess 'tails' or 'bubbles' (Fig. 2-16b). The discrete class of large DNA molecules and dimeric forms of this size class accounted for 68% of the total length of DNA, whilst the smaller heterodisperse, molecules both with and without 'tails' accounted for only 18% of the total length of DNA.

Thus it would appear that the larger component of the buoyant density analysis, of density 1.686 g/cm³, correlates with the major component of the electron microscope analysis, the class of molecules of about 8 µm in length, but a discrepancy still exists in the two sets of data.

B. **Detailed analysis of the closed-circular DNA band of a purified mitochondrial-enriched fraction**

In this preparation, the cells were not homogenised in a Braun shaker but rather were stripped of their outer walls using snail gut enzyme and then ruptured by passage through a French pressure cell (Appendix I). The crude mitochondrial pellet resulting was run on a 25-60% sucrose gradient and a band of mitochondrial particles was visible approximately half-way down the tube. A closed-circular DNA band was then obtained by running this purified mitochondrial fraction on a dye-buoyant density gradient and this DNA was analysed by both analytical ultracentrifugation and electron microscopy as before.

The analytical ultracentrifugation analysis revealed the presence of only a single DNA species of buoyant density 1.686 g/cm³ in the closed-circular DNA band isolated from
purified mitochondria (Fig. 2-17a). There was no trace of the second, minor DNA species of main-band buoyant density.

The electron microscope analysis revealed the presence of a small amount of linear DNA, 5% of the total length of DNA measured, which was randomly distributed in size from 0.4 to 7.2 μm. Length distribution analysis of the relaxed, closed-circular DNA molecules revealed the presence of a single class of DNA of mean length 8.2 μm (Fig. 2-17B) together with a few larger DNA molecules of dimeric length, (mean 16.0 μm).

Thus purification of the mitochondrial particles on a sucrose gradient has resulted in the complete elimination of all small circular DNA molecules, both with and without 'tails', from the closed-circular DNA band.

C. Detailed analysis of the small molecules present in the closed-circular DNA band of *H. wingei*

Having established that the small, circular DNA molecules do not occur in purified mitochondria of *H. wingei*, it was then of interest to question the nature of these molecules. It is possible that these small DNA molecules could have two origins, firstly, they could represent a form of plasmid DNA occurring in the cytoplasm of the cell, similar to the situation found with O DNA in *S. cerevisiae*, or secondly, they could represent intermediates in the replication of chromosomal DNA that appear in the closed-circular DNA band after cell homogenisation.

For this detailed analysis, a closed-circular DNA band was obtained from a crude mitochondrial-enriched
Analysis of the closed-circular DNA band isolated from a purified mitochondrial-enriched fraction of *H. wingei*. The analysis is by both analytical ultracentrifugation (A) and electron microscopy (B).

A, represents the buoyant density trace viewed in the analytical ultracentrifuge. Only a single DNA peak of buoyant density 1.686 g/cm³ is apparent. *M. luteus* DNA of density 1.731 g/cm³ is a marker.

B, represents the length distribution of the relaxed circular DNA molecules viewed in the electron microscope. A major peak, n = 94 molecules, l = 8.16 ± 0.20 µm, is apparent and so are some larger molecules, which are dimers of the monomer peak, n = 4 molecules, l = 15.98 ± 0.21 µm. No circles smaller than 7.8 µm either with or without 'tails' were viewed.
fraction, which once again contained both the lighter and heavier buoyant density DNA species in approximately equal amounts. For the electron microscope analysis the grid was not scanned randomly, but areas containing small relaxed DNA molecules were photographed preferentially and those small molecules with 'tails' were ignored in the analysis. The resulting histogram (Fig. 2-18) shows the appearance of three separate size classes within the small DNA molecules present. The first class, comprising 53% by number of the small DNA molecules, is in the range of 0.2-1.6 μm with a mean length of 0.87 ± 0.31 μm; the second class representing 20% of the molecules, is in the range of 1.7-2.7 μm with a mean length of 2.06 ± 0.27 μm; and the last class representing 27% by number of the small molecules is in the range of 2.8-4.4 μm with a mean of 3.2 ± 0.34 μm. These classes are not a simple oligomeric series of the basic monomer length, 0.9 μm, and it is interesting to note that the second class, with a mean length of 2.06 μm, closely resembles the length of the φ DNA species found in Saccharomyces strains, although the distribution of its molecular lengths is broader than with φ DNA.

The question then arose whether the small circular molecules with 'tails' fell into the same size classes or whether they represented a completely different species of DNA molecule. In order to determine this, histograms of the small molecules both with and without 'tails' were drawn up from a closed-circular DNA fraction not previously photographed; the results are presented in Fig. 2-19. It is apparent that the same sizes as previously seen appear
Length distribution of relaxed circular DNA molecules from the closed-circular DNA band from a crude mitochondrial-enriched fraction of H. wingei, selectively photographed for small, circular DNA molecules. No molecules with double stranded DNA 'tails' attached have been included in this histogram. The total number of molecules in the large size class is 207, with an average length of 8.15 ± .39 μm. The total number of molecules in the small circular DNA region is 237 divided into 3 classes:

I - small size class: n = 122 molecules, l = 0.87 ± .31 μm

II - medium size class: n = 45 molecules, l = 2.06 ± .27 μm

III - large size class: n = 61 molecules, l = 3.20 ± .34 μm.
Length distribution of relaxed, circular DNA molecules from the closed-circular band of a crude mitochondrial-enriched fraction of *H. wingei*, selectively photographed for small, circular DNA molecules both with (A) and without (B) attached double stranded DNA 'tails'. The size classes seen in the small DNA molecules previously (Fig. 3-18) are indicated on the histogram (B). The number of molecules in the small circular regions are 149 (A) and 145 (B). The number of molecules in the large size class is 54 (B) with an average length of \( 8.79 \pm 0.37 \mu m \). The small circular DNA molecules with attached 'tails' (A) do not appear to fall into the size classes apparent in the small circular DNA molecules with no 'tail' attached (B).
FIG. 2-20

Length distribution of the attached double stranded DNA 'tail' on the small DNA molecules in Fig. 3-19A, expressed as a percentage of the parent molecule to which the 'tail' is attached. The number of molecules in the figure is 149. The graph is a plot of the length of the parent molecule (µm) against the length of the 'tail' expressed as a percentage of the parent molecule.
in the classes of small DNA molecules without 'tails' (Fig. 2-19b), and account for: Class I - 57%, Class II - 15% and Class III - 28%. In this sample, it appears that there might be more than a single class of molecules in the smallest group, Class I: 0.2-1.6 μm. The small DNA molecules with 'tails' do not appear to fall into the same classes as those molecules without 'tails' and the distribution of size lengths appears to fall off gradually from the smallest to largest molecule. The length of the tail can also be expressed as a percentage of the length of the parent molecule. Such a plot for these small DNA molecules with 'tails' shows that the great majority of the DNA molecules have 'tails' shorter than the length of the circular molecule to which they are attached (Fig. 2-20). However, some 14% of the 'tails' are longer than the parent molecule, some up to 5 times as long, but all of these circles are less than 1 μm in length with the majority being about 0.5 μm in length.

Discussion

The results presented in this Section have resolved the complex situation present in the closed-circular DNA band from the petite-negative yeast H. wingei. The analyses of the closed-circular DNA band both before and after purification of the mitochondria, show unequivocally that the large size class of DNA molecules about 8.2 μm in length, has a buoyant density of 1.686 g/cm³ and represents the mt DNA of this yeast species. Further, the purification of the mitochondria on a sucrose gradient resulted in the
coincidental elimination of both the small DNA circles and the peak of main-band buoyant density from the closed-circular DNA band. Thus if any small DNA molecules of mitochondrial buoyant density exist, they do not occur in the mitochondria. This then makes it likely that all of the small DNA molecules are of main-band buoyant density.

The length analysis of the small DNA circles without 'tails' reveals the presence of 3 or 4 discrete size classes. These size classes are not oligomeric repeats of a basic monomer unit and are present in approximately the same proportion in different preparations. The lowest frequency size class has the same length as o DNA, about 2 μm.

The small, circular DNA molecules with double-stranded 'tails' are not replicating intermediates of mt DNA in H. wingei as originally thought. Rather, it is felt that they represent some form of replicating chromosomal DNA that appears in the crude mitochondria-enriched fractions. The lengths of the 'tails' are shorter than the parent molecule, except in the case of the very small DNA circles of less than 1 μm, when the 'tail' can be up to 5 times the length of the parent molecule.

In conclusion, it has been confirmed that the mt DNA of H. wingei is approximately one-third the size of that from the petite-positive yeast S. cerevisiae but has a similar buoyant density. Further, the small DNA circles viewed are not found in the mitochondria and appear to be of main-band buoyant density. Thus it is probable that
the small circular DNA represents both chromosomal replicating intermediates lacking the double-stranded 'tails' and a form of plasmid DNA resembling \( \sigma \) DNA which is known to be present in both petite-positive and petite-negative species of the genus *Saccharomyces*. 
General discussion

There exist areas associated with mitochondrial functions in which petite-positive and petite-negative yeasts differ. Petite-negative species are generally characterized by their limited ability to grow anaerobically (Bulder 1964A; Subik et al. 1974) and by their failure to exhibit the phenomenon of catabolite repression (glucose repression of respiration) (De Deken 1966; McClary and Bowers 1967, 1968). This effect is usually measured by RQ measurements [ratio of glucose fermented (measured by CO₂ evolved) to glucose oxidised (measured by O₂ uptake)]; this ratio is high in glucose-repressed petite-positive species. This lack of glucose repression has been reported for the petite-negative species studied here, that is H. wingei (Crandall 1973B), C. parapsilosis (Kellerman et al. 1969; Bulder 1964B; Mahler and Perlman 1971) and K. lactis (Luha et al. 1971). However, the fourth petite-negative species, S. pombe, does show glucose repression of mitochondrial enzymes (Heslot et al. 1970A; Poole and Lloyd 1973), with both super-repressed and derepressed mutants having been isolated (Foury and Goffeau 1972), and also grows for at least fourteen generations anaerobically. For these reasons, a lot of people regard S. pombe as intermediate between a petite-positive and petite-negative yeast. However, no cytoplasmically-inherited petite mutants have been isolated from S. pombe (Heslot et al. 1970B).

The suggestion that petite-negative yeasts could not form viable petite mutants because such species were unable to obtain sufficient energy for growth by fermentation
(Bulder 1964A), is invalidated for three reasons. Firstly, De Deken (1966) demonstrated that a number of petite-negative species could grow in a concentration of acriflavine which was sufficient to completely inhibit respiratory enzyme synthesis. Secondly, Subik et al. (1974) demonstrated that the ability of a cell to grow under strictly anaerobic conditions is not related to its respiration or fermentation capacities and that these capacities are also not directly responsible for the ability of some species to form respiration-deficient mutants. Thirdly, viable chromosomally-inherited, respiratory-deficient mutants have been isolated from a number of petite-negative yeasts (Crandall 1970B; Heslot et al. 1970B; Del Guidice and Puglisi 1974).

The response of petite-negative yeasts to the petite-inducing agent ethidium bromide appears to differ, although no cytoplasmically-inherited, respiratory-deficient mutants are induced. In some species the effects of the drug are reversible, implying an inhibition of mt DNA transcription, whilst in others the drug is lethal, which is not due to insufficient fermentation capacity as is usually assumed (Subik et al. 1974). Reports conflict about the presence or absence of mt DNA in whole cell homogenates from petite-negative yeasts cultured for several generations in EtBr. Some reports have shown a complete loss of mt DNA under such conditions in the Kluyveromyces species K. lactis (Luha et al. 1971) and K. fragilis (Luha et al. 1974), whilst another report shows no diminution in amount or alteration in buoyant density for the mt DNA from S. pombe
under the same conditions (Bandlow and Kaudewitz 1974). The way in which EtBr and other agents induce the petite mutation is not known, but hypotheses abound (Mahler and Perlman 1972; Mahler and Bastos 1974). However, as suggested by Clark-Walker and Miklos (1974B), these agents may simply exacerbate a spontaneous process occurring in high frequency.

Of the six petite-negative yeasts studied, five had mt DNA of higher buoyant density than that of *S. cerevisiae*, whilst the sixth, *H. wingei* has mt DNA of only slightly higher buoyant density (1.686 g/cm³). On the basis of this buoyant density difference, it is quite possible that the mt DNA of the former petite-negative yeasts lack the AT-rich sequences associated with sites of partial homology within the mitochondrial genome, and cannot undergo the same excision-insertion processes as shown by *S. cerevisiae* mt DNA.

In the case of the latter yeast, *H. wingei*, and three other petite-negative yeasts studied by others *K. fragilis* (mt DNA 1.683 g/cm³) (Luha et al. 1974), *C. utilis* (mt DNA 1.685 g/cm³) and *C. pseudotropicalis* (mt DNA 1.684 g/cm³) (Bak et al. 1969), all of which have mt DNA of buoyant density similar to that of *S. cerevisiae*, it is apparent that there is no direct correlation between the buoyant density of the mt DNA of a species and the response of the yeast species to petite-inducing agents.

The findings of a gross size difference between the mt DNA of the petite-positive yeasts *S. cerevisiae* and *S. carlsbergensis* and the four petite-negative yeasts outside of the genus, raises the possibility of structural and coding differences between the two sets of genomes. On a
size basis, the results would imply that the \textit{S. cerevisiae} mitochondrial genome has at least twice the coding capacity of the \textit{petite}-negative mt DNA. However, Bernardi and co-workers have shown that the mt DNA of \textit{S. cerevisiae} consists of an interspersion of AT-rich and GC-rich sequences and that the AT-rich sequences account for about 50\% of the mitochondrial genome. Further it is highly unlikely that these AT-rich sequences code for anything or that they are transcribed (Prunell and Bernardi 1974). Also it should be noted that much less than 10 x 10^6 daltons of the \textit{S. cerevisiae} mitochondrial genome has been accounted for in terms of known gene products (ribosomal and transfer RNAs). Thus it would appear premature to assume that the \textit{S. cerevisiae} mitochondrial genome necessarily contains more genetical information than those of \textit{K. lactis} and \textit{C. parapsilosis} (also see Chapter 3).

Little other data is available on the structure of the mt DNA of \textit{petite}-negative yeasts. In the case of \textit{K. lactis} mt DNA, there is no discrepancy between the G + C content as calculated by buoyant density or Tm (Smith \textit{et al.} 1968; Luha \textit{et al.} 1971). On the other hand, Bak \textit{et al.} (1969) have reported that the mt DNA of a series of \textit{Candida} species, all of which are probably \textit{petite}-negative, shows differences in the G + C content as calculated from the Tm and buoyant density data, resembling the discrepancies found in \textit{S. cerevisiae} mt DNA. In \textit{S. cerevisiae} these discrepancies were attributed to the presence of AT-rich sequences in the mt DNA (Bernardi \textit{et al.} 1970; Piperno \textit{et al.} 1972). This would suggest that either the mt DNA of these \textit{Candida}
species, and the other petite-negative yeasts of low buoyant density, may have AT-rich sequences or else these mt DNAs may be richer in A + T and this property is not associated with long AT-rich sequences.

Of relevance to this situation, the AT-rich sequences found in the mt DNA of *S. cerevisiae* have been shown to be rather large, between $1.6 \times 10^5$ – $2 \times 10^6$ daltons (Prunell and Bernardi 1974; Bernardi et al. 1972). In view of this, one can still postulate that structural considerations may be involved in petite negativity by suggesting that the AT-rich sequences are of insufficient length to provide sites of partial homology for internal cross-over events in the petite-negative mt DNA or else that the enzymes necessary for the internal cross-over events are absent in petite-negative yeasts. Also of relevance to this is that AT-rich sequences have also been reported in the mt DNA of *Euglena gracilis* (Fonty et al. 1975), *Drosophila melanogaster* (Bultmann and Laird 1973; Polan et al. 1973) and *Xenopus laevis* and *Xenopus mulleri* (Dawid 1972).

It has also been demonstrated that the small circular DNA seen in the closed-circular band of *H. wingei* is not present in purified mitochondria and appears to resemble either an o type DNA or a form of chromosomal replicating intermediate. However, this does not mean that the mt DNA of petite-negative yeasts cannot undergo such excision-insertion events, as one can postulate that the smaller genomes so produced may be defective for some gene or genes and hence be lethal.
Finally, in this Chapter, it has also been shown that there is no correlation between the presence of DNA and the response of the yeast species to petite-inducing agents.

In conclusion, a gross size difference exists between the mt DNA of *Saccharomyces petite*-positive species and that of other petite-negative species outside of the genus. Further studies on mt DNAs from petite-positive and petite-negative yeasts are necessary to determine the importance of structural difference in explaining the phenomenon of petite-negativity.
CHAPTER 3

STUDIES ON CLOSED CIRCULAR DNA ISOLATED FROM MITOCHONDRIAL-
ENRICHED FRACTIONS OF PETITE-POSITIVE YEASTS OTHER THAN IN
THE GENUS SACCHAROMYCES

SECTION 3-1

PRELIMINARY STUDIES ON PETITE-POSITIVE YEASTS OTHER
THAN IN THE GENUS SACCHAROMYCES
Introduction

The mechanism postulated independently by both Prunell and Bernardi (1974) and Clark-Walker and Miklos (1974B) to account for the high spontaneous frequency of petites, proposes that deletions occur from the circular mt DNA of *S. cerevisiae*. It is proposed that these deletions occur by way of internal recombination at sites of homology located in, or associated with, the AT-rich, non-coding sequences, which are heterogeneously distributed around the circular mitochondrial genome, resulting in the excision of smaller circular DNA molecules.

It is known that mt DNA from four petite-negative yeasts have circular genomes ranging from only a quarter to half the length of that of *S. cerevisiae* (O'Connor *et al.* 1975; Sanders *et al.* 1974; Chapter 2 of this thesis). This correlation between the smaller mitochondrial genome size and petite-negativity prompted the suggestion that one explanation for petite-negativity may be that these yeasts lack the AT-rich non-coding sequences and, by implication, the homologous sites which are thought to occur in the *S. cerevisiae* genome (Clark-Walker and Miklos 1974B; O'Connor *et al.* 1975).

As a continuation to testing this idea that there may be a correlation between genome size, presence of AT-rich sequences and the ability to form petites, the mt-DNA from a number of petite-positive yeasts, which are not closely related to *S. cerevisiae*, have been studied.

The early studies of Bulder (1964A) and DeDeken (1961, 1966), reported eight species of the genus *Saccharo-
myces and the single species of the genus *Nematospora* as petite-positive, ascomycetous yeasts (detailed in Appendix II). Of the asporogenous yeasts tested, only five species, *Torulopsis glabrata*, *T. holmii*, *Brettanomyces clausenii*, *Br. anomalus* and *Kloeckera africana* were found to be petite-positive (Appendix II). Most of the petite-positive species of the genus *Saccharomyces* are grouped in the *Saccharomyces sensu stricto* (Appendix II) and hence were felt to resemble closely *S. cerevisiae* and *S. carlsbergensis*, both of which belong to the same group. The ascomycetous, petite-positive yeast *Nem. coryli* is a plant pathogen not present in Australia and permission to import the species was refused (Appendix II).

Hence, only asporogenous, petite-positive yeasts were available for study. However, out of the five species reported, *T. holmii* is the imperfect form of the ascomycetous, petite-positive yeast *S. exiguus* (Lodder 1970) and was therefore rejected from the present study. This left four asporogenous petite-positive yeasts, these being *Br. anomalus*, *Br. clausenii*, *T. glabrata* and *Kl. africana*.

There are seven species in the genus *Brettanomyces* (van der Walt 1970B) of which two species have been reported as petite-positive and two as petite-negative (Appendix II). *Brettanomyces* is the asporogenous form genus of *Dekkera* and the two genera differ only in that the latter genus shows alternation of generations with the formation of asci and ascospores. Thus it was felt to be of interest to study the two species of the genus, *D. bruxellensis* and *D. intermedia*, as well as the asporogenous petite-positive yeasts previously detailed.
Materials and Methods

Strains

The six strains used in this study, *Br. anomalus*, *Br. clausenii*, *T. glabrata*, *Kl. africana*, *D. bruxellensis* and *D. intermedia*, were all obtained from the Centraalbureau voor Schimmelcultures and are detailed in Appendix I.

Methods

The methods for generation of EtBr-induced, respiratory-deficient mutants, and the preparation of both mitochondrial and closed-circular DNA, are detailed in Appendix I.

Results

Single colony, isolates of each strain, were used in all studies. However, *Br. clausenii* showed poor viability on arrival and rapidly died on storage either on slopes or silica gel. Because of this poor viability it was not considered worthwhile, at this stage, to obtain another culture.

A. EtBr-induced, respiratory deficient mutants

EtBr-induced, respiratory-deficient mutants were isolated from each of the five species by the technique described (Appendix I). Whilst it was found that an EtBr concentration of 2 mgs/ml was sufficient to prevent growth of the yeast colony through the central EtBr spot, it was decided to keep the same conditions as with *S. cerevisiae* strains and use an EtBr concentration of 10 mgs/ml in the central spot. Thus putative respiratory-deficient mutants of each species were isolated and tested for true respiratory-deficiency.
All putative respiratory-deficient isolates from four species grew on GYP but with the fifth species, *Br. anomalus*, it was found that 6 out of 10 isolates were inviable.

By dropping the putative respiratory-deficient isolates onto GLYYP plates, it was revealed that EtBr-induced respiratory-deficient mutants were isolated from each species at the frequencies of: (a) *T. glabrata*, 8 out of 10 isolates (other 2 isolates respiratory-sufficient), (b) *Kl. africana* 5 out of 5 isolates, (c) *D. bruxellensis*, 21 out of 21 isolates, (d) *D. intermedia*, 5 out of 5 isolates and (e) *Br. anomalus*, all four of the viable isolates were respiratory-deficient.

Thus it is apparent that EtBr induces respiratory-deficient mutants in all five of the species studied. These results confirm the previous observation that *T. glabrata*, *Kl. africana* and *Br. anomalus* are petite-positive (Bulder 1964A), and report that the two *Dekkera* species are also petite-positive.

B. Frequency of spontaneous, respiratory-deficient mutants

The frequencies of spontaneous, respiratory-deficient mutants of four of the five species were determined and are presented (Table 3-1). In each case, over four thousand colonies were scanned but in the case of *T. glabrata* it was found necessary to scan in the region of 300,000 colonies before definite respiratory-deficient mutants were isolated. With *Kl. africana*, the initial scan of approximately 5,000 colonies failed to reveal any spontaneous
TABLE 3-1

Frequency of spontaneous respiratory-deficient mutants of five petite-positive yeast species

<table>
<thead>
<tr>
<th>Species</th>
<th>Total number of putative RD mutants/total number of colonies</th>
<th>Number of putative RD mutants isolated</th>
<th>Number of viable isolates</th>
<th>Number of true RD isolates</th>
<th>Frequency of spontaneous RD mutants</th>
</tr>
</thead>
<tbody>
<tr>
<td>T. glabrata</td>
<td>19/300,000</td>
<td>19</td>
<td>15</td>
<td>4/15</td>
<td>0.001%</td>
</tr>
<tr>
<td>Br. anomalous</td>
<td>36/8004</td>
<td>24</td>
<td>17</td>
<td>3/17</td>
<td>0.056%</td>
</tr>
<tr>
<td>D. intermedia</td>
<td>125/6043</td>
<td>125</td>
<td>53</td>
<td>8/53</td>
<td>0.13%</td>
</tr>
<tr>
<td>D. bruxellensis</td>
<td>110/8932</td>
<td>110</td>
<td>30</td>
<td>5/30</td>
<td>0.056%</td>
</tr>
<tr>
<td>K1. africana</td>
<td>16/4890</td>
<td>16</td>
<td>2</td>
<td>0/2</td>
<td>&lt;0.02%</td>
</tr>
</tbody>
</table>
mutants and it is probably necessary to increase the number of colonies scanned, as was done with *T. glabrata*. The frequency of spontaneous respiratory-deficient mutants of *Kl. africana* remains to be determined.

It was found that all species had low frequencies of spontaneous, respiratory-deficient mutants, with *T. glabrata* having a frequency about a thousand-fold lower than most *S. cerevisiae* strains. The other species, *D. bruxellensis*, *D. intermedia* and *Br. anomalus* had frequencies similar to those found with *S. cerevisiae* strain Ure 3*R₂* (c.f. Chapter 4 of this thesis) which itself has a very low petite frequency for a *Saccharomyces* strain.

C. mt-DNA of the petite-positive species

Linear mt-DNA was isolated from all five species by centrifugation in EtBr-CsCl buoyant density gradients and analysed by analytical ultracentrifugation. The buoyant densities of the mt-DNAs are presented (Table 3-2).

It was found that the buoyant density of the mt-DNA of all five petite-positive species was low (high mole percent A + T) and similar to that found with *S. cerevisiae* mt-DNA. This result contrasts sharply with the situation in petite-negative yeast species, where it is found that five species have mt DNA buoyant densities much higher than *S. cerevisiae* and only one, *H. wingei*, had a buoyant density similar to that of *S. cerevisiae* mt DNA (Chapter 2).
### TABLE 3-2
Buoyant density data of the mt DNA of five petite-positive yeast species

<table>
<thead>
<tr>
<th>Determination number</th>
<th>Torulopsis glabrata</th>
<th>Brettanomyces anomalous</th>
<th>Dekkera intermedia</th>
<th>Dekkera bruzellensis</th>
<th>Kloecckera africana</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.6873</td>
<td>1.6834</td>
<td>1.6849</td>
<td>1.6862</td>
<td>1.6865</td>
</tr>
<tr>
<td>2</td>
<td>1.6866</td>
<td>1.6821</td>
<td>1.6856</td>
<td>1.6847</td>
<td>1.6826</td>
</tr>
<tr>
<td>3</td>
<td>1.6871</td>
<td>1.6842</td>
<td>1.6851</td>
<td>1.6867</td>
<td>1.6828</td>
</tr>
<tr>
<td>4</td>
<td>1.6879</td>
<td>1.6838</td>
<td>1.6856</td>
<td>1.6866</td>
<td>1.6800</td>
</tr>
<tr>
<td>5</td>
<td>1.6883</td>
<td>1.6852</td>
<td>1.6858</td>
<td>1.6861</td>
<td>1.6808</td>
</tr>
<tr>
<td>6</td>
<td>1.6884</td>
<td></td>
<td></td>
<td></td>
<td>1.6867</td>
</tr>
<tr>
<td>7</td>
<td>1.6862</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>1.6869</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>1.6879</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>1.6875</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>1.6874</td>
<td>1.6837</td>
<td>1.6854</td>
<td>1.6861</td>
<td>1.6832</td>
</tr>
<tr>
<td>Std. dev.</td>
<td>±0.0016</td>
<td>±0.0012</td>
<td>±0.0005</td>
<td>±0.0007</td>
<td>±0.0030</td>
</tr>
</tbody>
</table>
D. Closed-circular DNA from the petite-positive species

A closed-circular DNA band was isolated from the CsCl-EtBr buoyant density gradients of two of the five species, these being *T. glabrata* and *D. intermedia*. Initial studies on the other three species failed to reveal the presence of a closed-circular DNA band, but more recent work has shown that a closed-circular DNA band can be isolated from two of these species, *Br. anomalus* and *D. bruxellensis* under certain conditions (Clark-Walker, private communication).

The analysis of the closed-circular DNA band of *T. glabrata* is dealt with in the following section.

Analytical ultracentrifugation analysis of the closed-circular DNA band of *D. intermedia* revealed the presence of a major DNA component of buoyant density similar to that of main-band DNA (1.702 g/cm³) plus a much smaller, broad peak of buoyant density similar to that of mt DNA (1.685 g/cm³) (Fig. 3-1 insert). Electron microscopy analysis of the same closed-circular DNA sample showed the presence of a number of small, apparently-heterodisperse DNA molecules (Fig. 3-1). There appears to be at least 3 size classes of small DNA molecules but there is no size class of larger molecules to account for the minor peak of mt DNA buoyant density. The analysis of this closed-circular DNA was not pursued as in each preparation to date, the DNA peak of mitochondrial buoyant density seen in the analytical ultracentrifuge traces has always been a very small, broad peak.

Discussion

It is known that some yeasts from widely separated
Fig. 3-1

(A) Length distribution of relaxed closed-circular DNA molecules from the closed-circular DNA band of *Dekkera intermedia*. Total number of molecules measured is 281.

(B) Uviscan trace of the CsCl buoyant density gradient in the analytical ultracentrifuge of the same closed-circular DNA band of *D. intermedia* as was analysed in the electron microscope. DNA from *Micrococcus luteus* at a buoyant density of 1.731 g/cm$^3$ is a marker.
Number of molecules

<table>
<thead>
<tr>
<th>Length of molecules (μm)</th>
<th>Density (g/cm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.685</td>
</tr>
<tr>
<td>2</td>
<td>1.702</td>
</tr>
<tr>
<td>4</td>
<td>1.731</td>
</tr>
</tbody>
</table>

Absorbance

Density →

Number of molecules →
genera are capable of giving rise to respiratory-deficient mutants similar in phenotype to petites from *S. cerevisiae*. Amongst these yeasts are *T. glabrata*, *Kl. africana* and *Br. anomalus*, all of which have previously been reported to be petite-positive (Bulder 1964A; DeDeken 1961, 1966).

The genus *Brettanomyces* is the asporogenous form genus of *Dekkera* (Van de Walt 1970B) and the results show that the two species in the genus, *D. bruxellensis* and *D. intermedia* are also petite-positive, yielding viable respiratory-deficient isolates on treatment with EtBr. This is the first time it has been reported that *Dekkera* species are petite-positive.

The frequencies of spontaneous respiratory-deficient mutants are low in all five species. (The frequency in *Kl. africana*, which remains to be properly determined, is probably less than 0.02%). One of the species, *T. glabrata*, has a frequency which is a thousand-fold lower than most *S. cerevisiae* strains. This would imply a major difference in structure between the mt DNA of *T. glabrata* and *S. cerevisiae*. Similarly, although the other species have higher frequencies than *T. glabrata*, they are low compared to *S. cerevisiae*, again implying a difference in structure between the mt DNA of these species and that of *S. cerevisiae*.

A question that arises is whether these respiratory-deficient mutants are true cytoplasmic petites or not. In view of the lack of genetic systems available in either the ascomycetous genus *Dekkera* or the asporogenous yeasts, the cytoplasmic nature of these mutants could be determined by isolating the closed-circular mt DNA. In the case of EtBr-induced petites of *S. cerevisiae*, a high concentration
of EtBr eliminates all detectable mt DNA (Goldring et al. 1970; Nagley and Linnane 1970). Hence, buoyant density analysis of the mt DNA of the EtBr-induced mutants should show whether these are true petites or not. As discussed in the following section, the EtBr-induced respiratory-deficient mutants of *T. glabrata* lack all detectable mt DNA, indicating that the mutants are cytoplasmic in origin.

The buoyant density of the mt DNA of all five petite-positive species is low (high mole percent A + T) and similar to that found in the petite-positive *Saccharomyces* species. This high A + T content might suggest the occurrence of AT-rich sequences in these mt DNAs, similar to the situation found with *S. cerevisiae* mt DNA (Bernardi et al. 1970). This result contrasts sharply with the petite-negative yeasts, where of the six species studied, five had mt DNA of higher buoyant density than *S. cerevisiae*, whilst the sixth had mt DNA of similar buoyant density (Chapter 2).

The closed-circular DNA band from a mitochondrial enriched fraction from *D. intermedia* contained a major DNA species of main-band buoyant density and a very small, broad peak of mt DNA buoyant density. This profile has also been seen with the closed-circular DNA band from wild-type strains of *S. cerevisiae* and always correlated with the presence of 'fish-net' complexes in the electron microscope analysis (O'Connor and Clark-Walker, unpublished results). These fish-net structures were also present in the closed-circular band of *D. intermedia*. The mt DNA of a low-buoyant density petite *RD^A* is a perfect repeating unit of 70 nucleotides long but occurs in the cell in the
form of large fish-net complexes (Hollenberg et al. 1972). Thus it appears that these fish-net complexes are composed of mt DNA and are isolated in the closed-circular DNA band.

The electron microscope analysis of the closed-circular DNA band of *D. intermedia* revealed the presence of a number of small DNA molecules, heterodisperse in length distribution. There was no sign of a larger DNA class of molecules. These heterodisperse, small DNA molecules of main-band buoyant density resemble the situation found in *H. wingei* and *C. parapsilosis* (Chapter 2). By contrast, in *S. cerevisiae* the $o$ DNA component has a buoyant density similar to that of main-band DNA but the electron microscope analysis shows it to be composed of two distinct DNA species of differing length (Clark-Walker 1973). The large species of $o$ DNA of length 2.8 $\mu$m, is thought to have a higher buoyant density of 1.704 g/cm$^3$ (Clark-Walker and Miklos 1975B). Thus only the $o$ DNA component of length 1.9 $\mu$m has a buoyant density of main-band DNA (1.701 g/cm$^3$), similar to the situation found with the petite-negative yeast *S. rouxii* (Section 2-2). It is of interest to note that the major DNA species in the electron microscope analysis of the closed-circular DNA of *D. intermedia* also has a length of about 2 $\mu$m and a buoyant density similar to that of main band DNA.

The characterization of the closed-circular DNA band isolated from mitochondrial-enriched fractions of the wild-type and respiratory-deficient mutants of *T. glabrata* are presented in the following Section.
SECTION 3-2

RESPIRATORY-DEFICIENT MUTANTS OF TORULOPSIS GLABRATA, A
YEAST WITH CIRCULAR MITOCHONDRIAL DNA OF 6 µm
Introduction

In the previous Section it has been shown that **Torulopsis glabrata** is a petite-positive yeast with a very low frequency of spontaneous respiratory-deficient mutants and mt DNA of low buoyant density. Preliminary studies showed that it was possible to isolate a closed-circular DNA band of mitochondrial DNA buoyant density. Therefore, it was decided to investigate this yeast in some detail in regard to its mt DNA and the changes occurring to this DNA in spontaneous mutants.

Materials and Methods

**Organism.** *T. glabrata* strain 138 was obtained from the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands, and maintained on GYP slopes.

**Methods.** The media used are detailed in Appendix I. The techniques used for the isolation of spontaneous respiratory-deficient mutants for the determination of growth rates, absorption spectra, DNA renaturation kinetics and DNA sedimentation values and for the characterization and purification of closed-circular DNA, are all detailed in Appendix I.

Results

A. **Closed-circular DNA from the wild-type and EtBr-induced respiratory-deficient mutants**

Visualization by UV light of the CsCl-EtBr buoyant density gradients of mitochondria-enriched fractions revealed the presence of three DNA bands in the wild-type strain but only two DNA bands in the EtBr-induced, respiratory-deficient mutants of *T. glabrata* (photo 3-1).
Caesium-chloride, ethidium-bromide buoyant density gradients of mitochondria-enriched fractions of the wild-type (1) and an EtBr-induced respiratory-deficient mutant e[rho]−4 (2) of *T. glabrata*. The photograph is taken through an orange, UV filter (λ = 350 nm). Two top linear DNA bands and a lower, closed-circular DNA band are visible in the wild-type strain (1), whilst only a single top linear DNA band and a very faint, lower closed-circular DNA band are present in the EtBr-induced, respiratory-deficient mutant (2). The top DNA band and closed-circular DNA band fractions were removed by side-puncture for analysis.

The band near the bottom of the wild-type strain(1) is a non-fluorescent, light-scattering band of carbohydrate, whilst the large amount of fluorescence at the bottom of the tubes is RNA (c.f. photo 4-2).
CsCl-EtBr gradients of the EtBr-induced, respiratory-deficient mutants contained a faint lower closed-circular DNA band.

DNA molecules viewed in the electron microscope from the closed-circular DNA band of the wild-type strain are illustrated (Fig. 3-2.1) and the size distribution of the molecules presented (Fig. 3-3.1). Two discrete size classes of circles are present with lengths near 3 and 6 μm. Some circular molecules with lengths less than 3 μm and greater than 6 μm are also present. The molecular weights of the two major components have been determined by spreading the closed-circular DNA in the presence of circularised λ DNA, (Fig. 3-2.1). From a comparison of the circular molecules relative to the size of the λ DNA, a molecular weight of 6.6 for the smaller and 12.8 x 10^6 daltons for the larger circles, have been determined (Fig. 3-3.2). Analysis of the closed-circular DNA of the wild-type strain by analytical ultracentrifugation revealed the presence of three peaks with buoyant densities of 1.710, 1.701 and 1.686 g/cm^3 (Fig. 3-4, Table 3-3).

Length distribution of the DNA molecules viewed in the electron microscope from the closed-circular DNA band from the EtBr-induced, respiratory-deficient mutants, revealed the loss of the large circular molecules from the histograms (Fig. 3-3.3 and 3-3.4) in all four mutants studied. Buoyant density analysis of the closed-circular DNA bands from the EtBr-induced respiratory-deficient mutants revealed the coincident loss of the 1.686 g/cm^3 buoyant density peak (Fig. 3-4). Hence, an assignment of the 1.686 g/cm^3 component to the 12.8 x 10^6 daltons size
Fig. 3-2

Molecules from the closed circular DNA preparations from respiratory competent wild-type (1) and two respiratory deficient mutants s[rho]$^{-3}$ (2) and s[rho]$^{-19}$ (3) of *T. glabrata*. Circular molecules of the 3 μm and 6 μm size classes, together with a circular λ DNA (arrowed) are illustrated in (1). The figures are at a magnification of 22,850, the bar represents 1 μm.
Length distribution of relaxed circular molecules from the closed circular DNA preparations from respiratory competent wild-type \textit{T. glabrata} spread in the absence (1) and presence (2) of circularised \textit{\lambda} DNA (peak III) and from two ethidium bromide induced respiratory deficient mutants \textit{e[rho]}\textsuperscript{-1} (3) and \textit{e[rho]}\textsuperscript{-4} (4). The number of molecules, mean lengths and standard errors of each peak have been included in the Figure.
Traces of CsCl buoyant density gradients in the analytical ultracentrifuge. Each trace is of the closed circular DNA fraction from the *T. glabrata* strain indicated at the left of the figure. DNA from *Microccocus luteus* at a buoyant density of 1.731 g/cm³ is a marker.
TABLE 3-3

Buoyant densities of the DNA components present in the closed-circular DNA band isolated from the wild-type, EtBr-induced and spontaneous mutants of

*T. glabrata*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Number of determinations</th>
<th>Buoyant densities of DNA components in the closed circular DNA (g/cm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>8</td>
<td>1.6856 ± .0008 1.7009 ± .0011 1.7096 ± .0007</td>
</tr>
<tr>
<td>e[rho]⁻¹</td>
<td>2</td>
<td>None detectable 1.7009</td>
</tr>
<tr>
<td>e[rho]⁻⁴</td>
<td>2</td>
<td>None detectable 1.7016</td>
</tr>
<tr>
<td>s[rho]⁻³</td>
<td>1</td>
<td>1.6818 1.6995 1.7093</td>
</tr>
<tr>
<td>s[rho]⁻¹⁰</td>
<td>1</td>
<td>1.6805 1.7010 1.7085</td>
</tr>
<tr>
<td>s[rho]⁻¹⁶</td>
<td>1</td>
<td>1.6801 1.7007 1.7106</td>
</tr>
<tr>
<td>s[rho]⁻¹⁹</td>
<td>2</td>
<td>1.6784 1.7015 1.7103</td>
</tr>
</tbody>
</table>
class of DNA can be inferred. Although the large circle size class is lost from all four ethidium bromide-induced mutants (Table 3-4), two mutants, e[rho]⁻¹ and e[rho]⁻⁴ are illustrated as they have been consistently found to differ both in the ratios of 1.701 to 1.710 g/cm³ buoyant density components in their closed circular DNAs (Fig. 3-4), and also in their absorption spectra (see below). The reason for these differences is unknown. Additionally the small number of circular molecules in the larger size class seen in Figs. 3-3.3 and 3-3.4 are most probably dimers of the 3 µm DNA species rather than remnants of the 1.686 g/cm³ - 12.84 x 10⁶ dalton molecules.

When mitochondria are banded on a sucrose gradient only the 1.686 g/cm³ DNA is recovered, suggesting that the mitochondrial genome is located on the 6 µm DNA molecules. An independent estimate of the mitochondrial genome size was made by renaturation kinetic analysis. The second order rate plot for the 1.686 g/cm³ DNA is shown in Fig. 3-5. The renaturation rate constant is 345 L mole⁻¹ sec⁻¹ at 60°, which is approximately Tm-25° in 1M NaCl (Clark-Walker, unpublished observations). From this value a kinetic complexity of 14.8 x 10⁶ daltons is obtained using the determined S²⁰,W value of 6.05. As this sample is contaminated with approximately 7% nuclear buoyant density DNA, and as the renaturation rate is affected by base composition, being slower for more AT-rich DNAs (Wetmur and Davidson 1968), the above result is in reasonable agreement with the molecular weight determined by electron microscopy and indicates that the 6 µm circles are unique.

A tentative buoyant density for the 3 µm circular
Fig. 3-5

Second order rate plot for the renaturation of the 1.686 g/cm$^3$ buoyant density DNA from $T. glabrata$. 
DNA can be inferred from the correlation which exists between the amount of the 1.710 g/cm$^3$ component and the number of 3 μm circular DNA molecules in the two respiratory deficient isolates, e[rho]$^{-1}$ and e[rho]$^{-4}$. With e[rho]$^{-4}$ the 1.710 g/cm$^3$ DNA is 63% of the total (Table 3-4) and by electron microscopy the 3 μm circular molecules and oligomers account for 76% of the total length of all molecules, including smaller circles and linear DNA. On the other hand, with e[rho]$^{-1}$ only 27% of the DNA is the 1.710 g/cm$^3$ peak, while the 3 μm circular molecules and oligomers account for only 30% of the total length of DNA. These results suggest that the 3 μm circular DNA size class has a buoyant density of 1.710 g/cm$^3$.

B. Isolation of spontaneous respiratory-deficient mutants

Initially, no spontaneous respiratory-deficient mutants were found when a few thousand colonies of wild-type *T. glabrata*, grown in EtOH YP, were plated onto the selective medium plates, GGYP. That this observation reflects a low frequency of such mutants was clarified by plating a mixture of respiratory competent cells and ethidium bromide induced respiratory deficient mutants. As seen in Fig. 3-6, the two colony types can be easily distinguished. Subsequently nineteen spontaneously arising putative respiratory deficient colonies were selected on the basis of small size after scanning 3 x 10$^5$ colonies (Table 3-5). Of the initial nineteen isolates, four were inviable, one grew slowly on both glycerol and glucose media, four showed only large colonies on GGYP medium, nine showed both large
TABLE 3-4

Proportions of buoyant density components in the closed circular DNA preparations from four ethidium bromide induced respiratory deficient isolates of *T. glabrata*

<table>
<thead>
<tr>
<th>Mutant number</th>
<th>Buoyant density component</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.686 g/cm³</td>
</tr>
<tr>
<td>Wild-type</td>
<td>57%</td>
</tr>
<tr>
<td>e[rho]⁻¹</td>
<td>None detectable</td>
</tr>
<tr>
<td>e[rho]⁻²</td>
<td>None detectable</td>
</tr>
<tr>
<td>e[rho]⁻³</td>
<td>None detectable</td>
</tr>
<tr>
<td>e[rho]⁻⁴</td>
<td>None detectable</td>
</tr>
</tbody>
</table>
Fig. 3-6

Large respiratory competent and small ethidium bromide induced respiratory deficient mutant colonies of *T. glabrata* growing on selective GGYP medium. Note the smooth margins of the large colonies.
### TABLE 3-5

Putative spontaneously-arising respiratory deficient mutants of *T. glabrata*

<table>
<thead>
<tr>
<th>Isolate number</th>
<th>First subculture</th>
<th>Second subculture</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Colony morphology on GGYP</td>
<td>Growth on GLYYP</td>
<td>Colony morphology on GGYP</td>
</tr>
<tr>
<td>1</td>
<td>large +</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>no growth -</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>mixed + small</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>mixed + mixed +</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>mixed + small +</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>large +</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>no growth -</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>large +</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>very small +(v. slow)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10*</td>
<td>mixed + small</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>no growth -</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>mixed + mixed +</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>mixed + small +</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>large +</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>mixed + mixed +</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16*</td>
<td>small - small</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>no growth -</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>mixed + mixed +</td>
<td></td>
<td></td>
</tr>
<tr>
<td>19*</td>
<td>mixed + small</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

+ Growth
- No growth
* Isolates retained for further analysis
and small colonies on this medium and only one $s{\text{rho}}^{-16}$, was composed entirely of respiratory deficient cells. Subsequently one small colony was selected for retesting from each of the nine 'mixed' isolates on the GGYP plates. Three more purified respiratory deficient mutants $s{\text{rho}}^{-3}$, $s{\text{rho}}^{-10}$ and $s{\text{rho}}^{-19}$ were thus obtained, the remaining samples still possessing respiratory competent cells.

Because the four respiratory deficient isolates occurred at a frequency which suggested that they could arise from chromosomal gene mutations, and as genetic tests are as yet unavailable in asporogenous yeasts, other parameters were examined in order to decide between the cytoplasmic or chromosomal nature of the mutations. Although growth rates and absorption spectra were determined for each isolate (see below) the most revealing tests were analyses of the buoyant densities and sizes of the closed circular DNAs.

C. Closed-circular DNA from the spontaneous, respiratory-deficient mutants

Closed-circular DNA was isolated from CsCl-EtBr-buoyant density gradients from mitochondria-enriched fractions from each of the four spontaneous, respiratory deficient mutants and analysed by both analytical ultracentrifugation and electron microscopy.

Buoyant density analyses of the closed-circular DNA bands showed, in addition to the 1.710 and 1.701 g/cm$^3$ components, a lighter component in each isolate with a lower buoyant density than in the wild-type (Fig. 3-4,
Examination of the closed-circular DNAs from each mutant by electron microscopy showed the presence of a large number of small circular DNA molecules (Figs. 3-2.2 and 3-2.3). With $s(rho)^{-3}$ the size of the molecules fell into an oligomeric series based on the length of the smallest circles of 0.42 μm (Fig. 3-7.1). The situation is more complex with the other mutants. The isolate, $s(rho)^{-10}$, in addition to having circular DNA molecules which fall into an oligomeric series based on a monomer length of 0.41 μm, also appears to have at least one subset series of molecules indicated by arrows at a and b (Fig. 3-7.2). The two other mutants, $s(rho)^{-16}$ and $s(rho)^{-19}$ contained a large number of very small circular DNA molecules with no clearly defined size classes, although in $s(rho)^{-19}$ the peak indicated by an arrow at b, with a length of 0.41 μm, could be a dimer of the peak at arrow a with a length of 0.22 μm. This cannot be so for $s(rho)^{-16}$, because the peaks indicated by arrows at a and b have lengths of 0.20 and 0.34 μm respectively and the latter occurs in a higher frequency. Additionally in the mutant $s(rho)^{-3}$ peak VII, with a length of 3.3 μm is thought to be the circular DNA with a buoyant density of 1.710 g/cm³ which also occurs in the wild-type. Using a molecular weight of $6.6 \times 10^6$ daltons for this peak, a molecular weight of $0.85 \times 10^6$ daltons is obtained for the circular monomers in peak I. This value is 7.25% of the size of the original mitochondrial genome.

D. Other properties of the respiratory-deficient mutants

The growth rates of the spontaneous mutants, together with the two ethidium bromide induced mutants, are compared
Length distributions of relaxed circular molecules from the closed circular DNA preparations from the four spontaneously occurring respiratory deficient mutants s[ρ]^{-3} (1), s[ρ]^{-10} (2), s[ρ]^{-16} (3) and s[ρ]^{-19} (4) of *T. glabrata*. The peaks indicated by Roman numerals in 1 have numbers and mean lengths of: I, n = 48, \( \bar{l} = 0.42 \pm 0.01 \, \mu m \); II, n = 19, \( \bar{l} = 0.86 \pm 0.02 \, \mu m \); III, n = 14, \( \bar{l} = 1.38 \pm 0.008 \, \mu m \); IV, n = 6, \( \bar{l} = 1.80 \pm 0.02 \, \mu m \); V, n = 5, \( \bar{l} = 2.26 \pm 0.02 \, \mu m \); VI, n = 3, \( \bar{l} = 2.74 \pm 0.03 \, \mu m \); VII, n = 21, \( \bar{l} = 3.30 \pm 0.01 \, \mu m \) and in 2 have numbers and mean lengths of: I, n = 103, \( \bar{l} = 0.407 \pm 0.008 \, \mu m \); II, n = 54, \( \bar{l} = 0.88 \pm 0.01 \, \mu m \); III, n = 30, \( \bar{l} = 1.40 \pm 0.01 \, \mu m \); IV, n = 24, \( \bar{l} = 3.30 \pm 0.02 \, \mu m \). The peaks indicated by arrows at a and b in 2, 3, and 4 are described in the text. Note the ordinate scale change in panel 4.
against wild-type (Table 3-6). The growth of all respiratory deficient mutants are lower than the respiratory competent cells, but additionally the spontaneous mutants grow at a slower rate than the ethidium bromide induced mutants.

Absorption spectra of the wild-type, two ethidium bromide induced mutants and one spontaneous mutant, are illustrated in Fig. 3-8, where it is seen that the wild-type has absorption peaks with maxima at 606, 561 and 551 nm corresponding to the α bands for cytochromes aa₃, b and c respectively. The two ethidium bromide induced mutants differ slightly in their absorption spectra but nevertheless lack the 606 nm absorption peak of cytochrome aa₃ and most of the cytochrome b absorption band at 561 nm. Two small absorption peaks at 586 and 505 nm are now present in these spectra and could be due to protoporphyrin IX.

Only the absorption spectrum of the spontaneous mutant $s[\rho]^{-16}$ is illustrated, as all four isolates were identical. Like the ethidium bromide-generated mutants, these isolates all lack cytochromes aa₃ and most of the absorption band of cytochrome b. These parameters are compatible with a cytoplasmic origin for the spontaneous mutants.

Discussion

Circular DNA from a mitochondria enriched membrane fraction from respiratory competent wild-type T. glabrata contains three buoyant density peaks and three types of DNA circle sizes when observed in the electron microscope. The circular DNA of buoyant density of 1.686 g/cm³ and length of 6 μm which is present in purified mitochondria is absent
TABLE 3-6

Growth rates of the wild-type and respiratory deficient isolates of *T. glabrata*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Doubling time (min)</th>
<th>Doubling time (% of wild-type)</th>
<th>Final cell yield mgs/dry wt/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>56</td>
<td></td>
<td>4.4</td>
</tr>
<tr>
<td>e[rho]⁻¹</td>
<td>71</td>
<td>79%</td>
<td>2.4</td>
</tr>
<tr>
<td>e[rho]⁻⁴</td>
<td>65</td>
<td>86%</td>
<td>2.4</td>
</tr>
<tr>
<td>s[rho]⁻³</td>
<td>80</td>
<td>70%</td>
<td>2.4</td>
</tr>
<tr>
<td>s[rho]⁻¹⁰</td>
<td>80</td>
<td>70%</td>
<td>2.4</td>
</tr>
<tr>
<td>s[rho]⁻¹⁶</td>
<td>80</td>
<td>70%</td>
<td>2.4</td>
</tr>
<tr>
<td>s[rho]⁻¹⁹</td>
<td>84</td>
<td>67%</td>
<td>2.4</td>
</tr>
</tbody>
</table>
Absorption spectra of wild-type (1) two ethidium bromide induced mutants $e[^{\rho}o]^{-1}$ (2) and $e[^{\rho}o]^{-4}$ (3) and the spontaneously arising respiratory deficient mutant $s[^{\rho}o]^{-16}$ (4) of *T. glabrata*. Cell densities in the cuvettes in mg dry wt of cells/ml were: 1, 15.8; 2, 26.5; 3, 21.3; and 4, 26.8.
from ethidium bromide-induced respiratory deficient mutants. This is similar to the situation in *S. cerevisiae* whereby ethidium bromide can cause the formation of *petites* which have lost the 1.684 g/cm³–25 μm mitochondrial DNA (Goldring *et al.* 1970; Nagley and Linnane 1970).

The molecular weight of the mitochondrial DNA in *T. glabrata* has been estimated to be 12.8 x 10⁶ daltons by comparison with λ DNA used as an internal length standard. This value is supported by the estimate of 14.8 x 10⁶ daltons obtained from renaturation studies. This finding of the small size of the mitochondrial genome in *T. glabrata* has two implications or consequences in relation to larger mt DNA molecules. Firstly, it could imply that the size of larger molecules found in some yeasts, other lower eukaryotes and higher plants, may not necessarily be related to their coding function, and secondly, as discussed below, it raises the question of the origin of these larger molecules.

Another size class of circular DNA having a molecular weight of 6.65 x 10⁶ daltons must comprise most, if not all, of the 1.710 g/cm³ buoyant density peak. The circles with length below 3 μm and apparently heterodisperse in size, are similar to those found in some other yeasts (O'Connor *et al.* 1975). Their buoyant density has not been established, but they may in part be responsible for the small peak of 1.701 g/cm³ which is present in the supercoiled DNA of both wild-type and ethidium bromide-generated, respiratory-deficient mutants.

Spontaneously arising respiratory deficient mutants of *T. glabrata* are found to occur at the low frequency of
around $10^{-5}$. All four isolated mutants have mitochondrial DNA of altered buoyant density. This change is accompanied by the appearance of smaller circular molecules and the loss of the 6 μm size class. Two of the spontaneous isolates $s[\rho]^{-3}$ and $s[\rho]^{-10}$, have discrete circle size peaks falling into an oligomeric sequence reminiscent of the situation found with some ethidium-bromide-induced petites of *S. cerevisiae* (Locker et al. 1974). The other two isolates $s[\rho]^{-16}$ and $s[\rho]^{-19}$, have much smaller circular molecules with no clearly definable single monomer length. Heterodisperse circle sizes have also been observed in some petites of *S. cerevisiae* (Bernardi et al. 1968; Clark-Walker and Miklos 1974A), indicating that this is a general property of respiratory deficient isolates. The interesting question of whether these heterodisperse circular molecules co-exist within single cells is still undecided.

The occurrence of mitochondrial DNA of altered buoyant density and smaller circular size in the spontaneous mutants of *T. glabrata*, indicates that the mutants are cytoplasmic in origin. Additionally, the respiratory deficient phenotype in these mutants is accompanied by a loss of cytochrome aa$_3$ and a slower growth rate; properties characteristic of cytoplasmically inherited respiratory deficient mutants in *S. cerevisiae*. Although these results are strong arguments for the belief that the spontaneous mutants in *T. glabrata* are due to cytoplasmically located events, the possibility cannot at present be dismissed that these isolates carry additional chromosomal mutations affecting the respiratory competent phenotype.
The incidence of spontaneously occurring respiratory deficient mutants of *T. glabrata* is a thousand-fold less than in *S. cerevisiae*. This observation has a direct bearing on the proposed mechanism of formation of *petites* which invokes excisions from sites of homology thought to exist in, or be associated with, adenine and thymine-rich sequences (Prunell and Bernardi 1974; Clark-Walker and Miklos 1974B). The fact that AT-rich regions exist in the mitochondrial DNA of *T. glabrata* can be inferred from the broad range of the melting profile (Clark-Walker, unpublished observations) and the 1.678 g/cm³ buoyant density of the mitochondrial DNA from the isolate s[rho]⁻¹⁹. Therefore the thousand-fold lower incidence of spontaneously arising respiratory-deficient mutants in *T. glabrata* compared to *S. cerevisiae* is not due to the entire absence of AT-rich sequences in the mitochondrial DNA of the former yeast. Several factors need to be considered in attempting to explain the differing incidence of spontaneous mutants in the two species. Firstly, there may be more sites of homology in the mitochondrial DNA of *S. cerevisiae* than in *T. glabrata* simply because the former yeast has a genome four times the length of the latter. Additionally there may be a greater number of sites per unit length of DNA and these sites may differ in their size and the fidelity of sequence matching. Furthermore, the arrangement of the sites around the circular molecule may have an influence on whether sites can be physically brought into alignment for an internal recombination event to take place.

Pertinent to these points is the fact that the
mitochondrial DNA in *S. cerevisiae* is four times the length of that in *T. glabrata*. Furthermore some yeasts have circular mitochondrial DNAs either the same size as (*Schizosaccharomyces pombe*, 6 μm), or double (*Candida parapsilosis* 12 μm, *Kluyveromyces lactis* 12 μm) that of *T. glabrata* (O'Connor et al. 1975; Sanders et al. 1974). This could mean that the larger circular DNAs found in some yeasts are the result of one or two dimerizations, as suggested for the formation of the *Escherichia coli* chromosome (Zipkas and Riley 1975). Further support for the concept that the *S. cerevisiae* mitochondrial genome is the result of dimerization, comes from the finding that the sequences coding for the small and large mitochondrial ribosomal RNA subunits are separated by at least one third of the molecule (Sanders et al. 1975). This could either have occurred by transposition of one of the cistrons in the 25 μm molecule or more likely, it is postulated, by dimerization followed by devolution of redundant cistrons.

If indeed the mitochondrial DNA of *S. cerevisiae* has been formed from two rounds of dimerization then in addition to having sites of homology in non-coding AT-rich sequences, it could well have retained some sequence homology in other regions of the genome. Therefore the high frequency of spontaneously arising petites in *S. cerevisiae* may be the summation of two processes of excision, one occurring at sites in AT-rich regions, and the second at homologous sites in other regions of the genome. The second process may well not occur in *T. glabrata* because of the uniqueness of the genome and this could possibly be a further contributing factor to the low
frequency of spontaneous mutants in this yeast.

The present results are also crucial to an understanding of petite-negativity. It is now apparent that the smaller mitochondrial genome sizes in petite-negative yeasts, in comparison to *S. cerevisiae*, are not simply due to the absence of non-coding AT-rich sequences. Furthermore we have shown that one petite-negative yeast *Hansenula wingei*, with a circular mitochondrial DNA of 17.3 x 10^6 daltons, has the same buoyant density as in *T. glabrata* (O'Connor *et al*. 1975). This suggests, at least in the case of *H. wingei*, that failure to form viable respiratory deficient mutants may not be a consequence of its DNA structure. Other explanations need to be considered, such as the presence of viability in the mitochondrial genome (Clark-Walker and Miklos 1974B), or the absence of appropriate enzymes to catalyse the breakdown of the DNA.
CHAPTER 4

SUPPRESSIVENESS AND MITOCHONDRIAL DNA OF RECENTLY-ARISEN,
SPONTANEOUS PETITE-MUTANTS OF S. CEREVISIAE
Introduction

Suppressiveness is the ability of a [$\rho^-$] strain to transmit its petite genotype to zygotes resulting from a cross with a [$\rho^+$] strain of opposite mating type (Ephrussi et al. 1955, 1956; Ephrussi and Grandchamp 1966). A mutually repressive interaction between the 'suppressive factor' in the [$\rho^-$] strain and the 'normal genetic factor' in the [$\rho^+$] strain, was postulated to explain the phenomenon (Ephrussi et al. 1966).

Subsequent work has shown that suppressiveness is not so simple. The presence of mt DNA in the petite is necessary for suppressiveness but there is no apparent relationship between the buoyant density or coding capacity of the mt DNA and the degree of suppressiveness (Michaelis et al. 1971; Michels et al. 1974). Although the basic mechanism of this interaction between the [$\rho^-$] and [$\rho^+$] genotypes remains to be determined, Clark-Walker and Miklos (1974B) view suppressiveness as insertion of two circular molecules followed by subsequent excision from the hybrid molecule to produce defective genomes. Hence, suppressiveness is thought to have two components, the first is related to the amount of insertion that occurs between the [$\rho^-$] and [$\rho^+$] genomes and the second depends on the frequency of excisions from the partially duplicated molecules, which would itself depend on the size and genetic complexity of the mt DNA of the particular [$\rho^-$] strains involved.

Furthermore, if it is considered that the spontaneously-arising petite mutants result from an excision
process, then the frequency of such mutants may be a measure of the level of excision events in a particular strain. Thus it became of interest to determine if there was a correlation between the frequency of petite formation and the level of suppressiveness in individual strains.

An additional implication of the excision hypothesis is that spontaneous petites have lost different sectors of mt DNA. That this occurs has been convincingly demonstrated by the observation of complementation between different petites to yield respiratory competent [rho]+ cells (Clark-Walker and Miklos 1975A). Therefore it was also of interest to determine if recently-arisen, spontaneous petites showed any differences in their remaining mt DNA as judged from the parameters of buoyant density and suppressiveness.

Materials and Methods

Organisms

The four haploid strains of *S. cerevisiae* 4545, 410, 4342 and Ure 3.R2 were chosen on the basis of differing petite frequencies and also so that their mating-types and nuclear markers would complement, enabling selection of prototrophic diploids. These strains are detailed in Appendix I.

Another strain, 50Ra of spontaneous petite frequency 0.27%, was used in a single cross with petites from strain Ure 3.R2.
Methods

The selection of spontaneous petite isolates, the preparation of mt DNA and the determination of the degree of suppressiveness of the \([\text{rho}]^-\) isolates, are described in detail in Appendix I.

Results

A. Contamination of putative respiratory-deficient isolates by \([\text{rho}]^+\) colonies

Differences in colony morphology on GGYP plates could be detected after approximately 48 hours incubation at 30°C. A series of putative respiratory-deficient mutants were picked from each of the strains, subcultured onto 2% GYP and tested for \([\text{rho}]^+\) contamination by dropping aliquots onto GLY YP plates, as illustrated in photograph 4-1. It was found that the percentage of colonies contaminated with \([\text{rho}]^+\) cells varied not only from strain to strain, but also varied with the time at which colonies were picked (Table 4-1). When putative petites of the strains studied were picked at 48 hours, strain 410 with a high spontaneous petite frequency, showed no \([\text{rho}]^+\) contamination in the putative respiratory-deficient isolates, whilst strain Ure 3.R\(_2\), with a low spontaneous petite frequency, showed a very high level of \([\text{rho}]^+\) contamination. However, if the plates were incubated for a further 24 hours, and putative petites picked at 72 hours after plating the wild-type strain, the situation with strain 410 remains the same, but with strain Ure 3.R\(_2\) there is a dramatic drop in the \([\text{rho}]^+\) contamination level by 50%.
Putative respiratory-deficient mutants of

*S. cerevisiae* strain 4342 picked after 44 hours growth at 30°C on GGYP plates and dropped onto a GLY YP plate. The photograph shows three putative respiratory-deficient isolates with confluent [rho]+ contamination, a single isolate slightly [rho]+ contaminated and two isolates showing no [rho]+ contamination.
## TABLE 4-1

Contamination of putative respiratory-deficient isolates by [rho]$^+$ colonies

<table>
<thead>
<tr>
<th>Plate Incubation Time</th>
<th>48 hours</th>
<th></th>
<th>72 hours</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Putative R.D. mutants</td>
<td></td>
<td>Putative R.D. mutants</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Number/total number colonies</td>
<td>Number contaminated by [rho]$^+$/No. sampled</td>
<td>% [rho]$^+$ contamination</td>
</tr>
<tr>
<td><strong>S. cerevisiae Strain</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>410</td>
<td></td>
<td>101/1565</td>
<td>0/32</td>
<td>0%</td>
</tr>
<tr>
<td>4545</td>
<td></td>
<td>72/2744</td>
<td>6/24</td>
<td>25%</td>
</tr>
<tr>
<td>4342</td>
<td></td>
<td>37/3351</td>
<td>9/24</td>
<td>37%</td>
</tr>
<tr>
<td>Ure 3.R$_2$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. 8/5366</td>
<td></td>
<td>6/8</td>
<td>75%</td>
<td>0.04%</td>
</tr>
<tr>
<td>b. 24/4700</td>
<td></td>
<td>22/24</td>
<td>92%</td>
<td>0.05%</td>
</tr>
</tbody>
</table>
Thus it appears that with strain 410, the individual cells become completely petite very soon after plating and the picked colony consists of cells which are all respiratory-deficient. On the other hand, with strain Ure 3.R₂, the individual cells become petite at a much slower rate.

It is suggested that this is related to the rate of degeneration or decay of the mt DNA, being faster for strain 410 and slower for Ure 3.R₂. Accordingly, recently-arisen spontaneous petites were examined to see if the purported differences in the rate of decay of mitochondrial DNA produced differences in buoyant densities.

B. Buoyant density analysis of mt DNA from recently-arisen spontaneous [rho]⁻ isolates

The mt DNA was isolated by CsCl-EtBr buoyant density gradients (Appendix I and photo 4-2) from a mitochondria-enriched fraction from the recently-arisen spontaneous [rho]⁻ strains of three of the four strains, 4545, 4342 and 410. Unfortunately machine problems and time did not permit analysis of the mt DNA from spontaneous [rho]⁻ isolates of Ure 3.R₂.

The spontaneous petites from each strain were chosen for buoyant density analysis of mt DNA on the basis of wide differences in suppressiveness. It was found that in all three strains of S. cerevisiae, the buoyant density of the mt DNA of the spontaneous petite isolates was similar to that of the wild-type mt DNA (Table 4-2). Further, as reported previously (Section 1-2), the amount of mt DNA isolated from the spontaneous petites of strain 410 was
A photograph taken through an ultraviolet filter of fluorescent bands in sarkosyl lysates of mitochondria-enriched fractions after CsCl-EtBr buoyant density centrifugation for 48 hours at 48,000 r.p.m. and 10°C. The left-hand polyallomer tube contains a mitochondria-enriched pellet from petite 4342 s[rho]^{-5} and the right-hand tube contains a mitochondrial-enriched pellet from petite 4342 s[rho]^{-10}. Bands A and B are upper, linear DNA bands, band A being mt DNA and band B DNA of main-band buoyant density. Band C is the lower closed-circular DNA band and band D is a non-fluorescent, light-scattering band of carbohydrate. The large amount of fluorescence at the bottom of the tubes is RNA and the pellicle on the side of the tube is protein.
TABLE 4-2

Buoyant density data of the spontaneous [rho]^- isolates, s[rho]^- and the corresponding wild-type strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>% Suppressive</th>
<th>Number of determinations</th>
<th>Buoyant density of mt DNA g/cm^3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>vs 4545</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>vs 4342</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4545 [rho]^+</td>
<td>1%</td>
<td>3</td>
<td>1.6852 ± .0008</td>
</tr>
<tr>
<td>s[rho]^2</td>
<td>43%</td>
<td>1</td>
<td>1.6849</td>
</tr>
<tr>
<td>s[rho]^9</td>
<td>76%</td>
<td>1</td>
<td>1.6854</td>
</tr>
<tr>
<td>s[rho]^12</td>
<td>55%</td>
<td>3</td>
<td>1.6857 ± .0007</td>
</tr>
<tr>
<td>4342 [rho]^+</td>
<td>1%</td>
<td>3</td>
<td>1.6849 ± .0010</td>
</tr>
<tr>
<td>s[rho]^2</td>
<td>21%</td>
<td>2</td>
<td>1.6856 ave</td>
</tr>
<tr>
<td>s[rho]^5</td>
<td>28%</td>
<td>2</td>
<td>1.6851 ave</td>
</tr>
<tr>
<td>s[rho]^10</td>
<td>94%</td>
<td>2</td>
<td>1.6855 ave</td>
</tr>
<tr>
<td>410 [rho]^+</td>
<td>1%</td>
<td>3</td>
<td>1.6848 ± .0011</td>
</tr>
<tr>
<td>s[rho]^2</td>
<td>33%</td>
<td>1</td>
<td>1.6849</td>
</tr>
<tr>
<td>s[rho]^4</td>
<td>5%</td>
<td>1</td>
<td>1.6846</td>
</tr>
<tr>
<td>s[rho]^5</td>
<td>9%</td>
<td>1</td>
<td>1.6844</td>
</tr>
<tr>
<td>s[rho]^7</td>
<td>69%</td>
<td>1</td>
<td>1.6844</td>
</tr>
<tr>
<td>s[rho]^9</td>
<td>29%</td>
<td>1</td>
<td>1.6855</td>
</tr>
</tbody>
</table>
considerably reduced compared to the amount of mt DNA isolated from the wild-type strain under exactly the same conditions. The amount of mt DNA present in the petite isolates of 410 was so low that there was no top mt DNA band visible by excited fluorescence of the EtBr-CsCl gradients and only a very small amount detectable in the analytical ultracentrifuge. However, the small amount detectable was of unchanged buoyant density (Fig. 4-2). This situation is peculiar to strain 410 as with the other two strains, 4545 and 4342, it was possible to enrich for the mt DNA of the spontaneous petite isolates (Fig. 4-1, photograph 4-2).

C. Suppressiveness of recently-arisen spontaneous \([\rho^-]\) isolates

The strains used in this analysis consist of two of mating type \(\alpha\) (strains 4342 and Ure 3.\(R_2\)) and two of mating-type \(\alpha\) (strains 4545 and 410). A series of spontaneous \([\rho^-]\) isolates from each of the four strains were crossed to the two other strains of opposite mating type. From each cross the degree of suppressiveness was determined after plating onto GGSyVit plates. The results illustrated in Figs. 4-3, 4-4 have been expressed to the nearest 5%.

It is apparent that with three of the four series of petites, the degree of suppressiveness of individual isolates varies when crossed to the two strains of opposite mating-type. In the fourth case, 410 s\([\rho^-]\), there is no difference in the degree of suppressiveness of the individual
Uviscan traces of CsCl-buoyant density gradients in the analytical ultracentrifuge. The traces correspond to linear DNA bands of spontaneous petite isolates of *S. cerevisiae*:

(a) 4545 s[rho]$^{-12}$
(b) 4342 s[rho]$^{-5}$
(c) 4342 s[rho]$^{-10}$

DNA from *Micrococcus luteus* at a buoyant density of 1.731 g/cm$^3$ is a marker.
Uviscan traces of CsCl-buoyant density gradients in the analytical ultracentrifuge. The traces correspond to linear DNA bands from the wild-type (A) and spontaneous petite (B, C, D, E, F) strains of *S. cerevisiae* strain 410. The traces correspond to:

(A) $410 \rho^+$
(B) $410 \rho^{-2}$
(C) $410 \rho^{-4}$
(D) $410 \rho^{-5}$
(E) $410 \rho^{-7}$
(F) $410 \rho^{-9}$
Variations in suppressiveness of spontaneous petites from *S. cerevisiae* a strains when crossed to two or more strains of opposite mating type. The figures correspond to:

(A, B) petites from strain 4342 when crossed to strain 410 (A) and strain 4545 (B).

(X, Y, Z) petites from strain Ure 3.R₂ when crossed to strain 410 (X), strain 4545 (Y) and strain 50Ra (Z).

The degree of suppressiveness is expressed to the nearest 5%. The arrows represent the same petite crossed to two different strains.
Number of petites

(%) Suppressiveness

A

B

X

Y

Z
Variations in suppressiveness of spontaneous petites from *S. cerevisiae* a strains when crossed to two strains of opposite mating-type. The figures correspond to:

(A, B) petites from strain 410 when crossed to strain 4342 (A) and strain Ure 3.R₂ (B).

(X, Y) petites from strain 4545 when crossed to strain 4342 (X) and strain Ure 3.R₂ (Y).

The degree of suppressiveness is expressed to the nearest 5%. The arrows represent the same petite crossed to two different strains.
isolates when crossed to the two opposite mating type strains, but in both cases the isolates have low levels of suppressiveness.

The distribution of the suppressiveness profile of the spontaneous petites is skewed in all strains and hence the median value of the degree of suppressiveness, that point at which 50% of the spontaneous petites are on either side, was determined for each cross and are presented (Table 4-3).

The wild-type strains used in this suppressiveness analysis were chosen for their differing spontaneous petite frequencies. Amongst the strains used, there is almost a hundred-fold range in petite frequency from that of Ure 3.R₂ (0.13%) to 410 (9.3%). From Tables 4-1 and 4-3, it can be seen that a relationship exists between the median degree of suppressiveness of the petite isolates and the spontaneous petite frequency of the opposite wild-type strain to which the petites are crossed. For instance, arranging the opposite wild-type strains in order of decreasing spontaneous petite frequency (Table 4-3) correlates with a decrease in the median value of the degree of suppressiveness for all four series of spontaneous petites. This relationship is demonstrated more clearly by plotting the median value of the degree of suppressiveness against the spontaneous petite frequency of the opposite wild-type strain (Fig. 4-5). As the spontaneous petite frequency of the opposite wild-type strain decreases, the median value of the degree of suppressiveness decreases correspondingly.
Median values of suppressiveness of $s[rho]^{-}$ and $e[rho]^{o}$ petites when crossed to two strains of opposite mating-type. The figure in brackets after each strain is the spontaneous petite frequency of that strain.

<table>
<thead>
<tr>
<th></th>
<th>410 (9%)</th>
<th>4545 (2%)</th>
<th>50Ra (0.3%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4342 (1%)</td>
<td>1%</td>
<td>17%</td>
<td>2%</td>
</tr>
<tr>
<td>$s[rho]^{-}$</td>
<td>47%</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>$e[rho]^{o}$</td>
<td>2%</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4342 (1%)</td>
<td>10%</td>
<td>12%</td>
<td>10%</td>
</tr>
<tr>
<td>Ure 3.R$_{2}$ (0.1%)</td>
<td>$s[rho]^{-}$</td>
<td>57%</td>
<td>-</td>
</tr>
<tr>
<td>$e[rho]^{o}$</td>
<td>4%</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Median values of the degree of suppressiveness of spontaneous \( \text{[rho]}^- \) isolates (Table 4-3) plotted against the spontaneous petite frequency of the wild-type strain of opposite mating-type to which the isolates were crossed. The diagram represents the \( \text{[rho]}^- \) isolates from:

- \( \square - \square \) strain Ure 3.\( R_2 \)
- \( \bullet - \bullet \) strain 4342
- \( \triangle - \triangle \) strain 410
- \( \circ - \circ \) strain 4545
frequency of opposite strain

[Graph with data points and lines representing strains 4342 [ρ], 4545 [ρ], 410 [ρ], and URE3.R2.]

Median degree of suppressiveness %

Strains used
D. Variations in the median value of the degree of suppressiveness as a function of the time that the spontaneous [rho]- isolates are picked

Because it has been found that the ability to complement is a transient property of spontaneous petites (Clark-Walker and Miklos, 1975A), suggesting that ongoing changes occur in mt DNA, the suppressiveness of spontaneous petites was studied as a function of time. It was especially interesting to study this in strain 410 as a very rapid breakdown of the mt DNA in this strain has been implied earlier (Section 4(a)).

Putative [rho]- isolates from both strains 410 and 4545 were picked at various time intervals after plating onto GGYP plates, 44, 96 or 144 hours, crossed to the strains of opposite mating type and the suppressiveness patterns of the true petites observed.

Preliminary experiments with strain 410 showed that when picked after 44 hours growth on the GGYP plates, occasionally (2 times out of 7) the median value of the degree of suppressiveness of the true petite isolates was noticeably higher than the background level when crossed to strain Ure 3.R2, such a situation was always accompanied by an increase in the [rho]+ contamination of the picked, putative [rho]- isolates (Fig. 4-6).

A number of experiments were then performed where the putative [rho]- isolates were crossed at the same time, to both strains Ure 3.R2 and 4342. In the experiment illustrated (Fig. 4-7), the median level of the degree of
Variation in the distribution of the degree of suppressiveness of spontaneous petite isolates from *S. cerevisiae* strain 410 in two separate experiments when picked at 44 hours after plating and crossed to strain Ure 3.R₂. The median value of suppressiveness is 12% (A) with a [rho]⁺ contamination rate of 4% in the putative respiratory-deficient isolates, and 54% (B) with a [rho]⁺ contamination rate of 32% in the putative respiratory-deficient isolates. The degree of suppressiveness is expressed to the nearest 5%.
FIG. 4-7

Variations in suppressiveness of spontaneous $[\text{rho}]^-$ isolates from strain 410 when picked at different times after plating out and crossed to two opposite wild-type strains.

The histograms correspond to:

(A, B) $s[\text{rho}]^-$ picked at 44 hours, with a $[\text{rho}]^+$ contamination rate of 46%, crossed to strain 4342 (A) and strain Ure 3.R$_2$ (B).

(P, Q) $s[\text{rho}]^-$ picked at 91 hrs, with a $[\text{rho}]^+$ contamination rate of 0%, crossed to strain 4342 (P) and strain Ure 3.R$_2$ (Q).

(Z) $s[\text{rho}]^-$ picked at 166 hours, with a $[\text{rho}]^+$ contamination rate of 0%, crossed to strain Ure 3.R$_2$.

The degree of suppressiveness is plotted to the nearest 5%. The arrows represent the same petite crossed to two opposite strains.
suppressiveness is above background level at the earliest time of picking (44 hours) and is accompanied by an increase in the [rho]^{+} contamination of the putative [rho]^{-} isolates. Furthermore, when the background level of suppressiveness is subtracted from each cross, the median value of suppressiveness is about the same (25%) when crossed to both strains 4342 and Ure 3.R_{2}. This result is still in contrast to previous experiments.

When picked 48 hours later, at 92 hours after plating, the suppressiveness profile of the spontaneous petites is the same as seen before with all isolates grouped around the background level of suppressiveness when crossed to strain Ure 3.R_{2}, but slightly higher when crossed to strain 4342. This pattern is repeated at the later time point of 166 hours after plating, although only the cross to Ure 3.R_{2} is shown here. In other experiments the results are similar, that is if the general level of suppressiveness of the 410 spontaneous petites is above background level at the 44 hour point, then 48 hours later the suppressiveness of all isolates has fallen to the background level.

The same experiment was performed with strains 4545 using 20 putative [rho]^{-} isolates at various time intervals after plating the wild-type strain. It is apparent from Fig. 4-8 that the distribution of the degree of suppressiveness seen with the spontaneous petites is more widely dispersed than seen with petites from strain 410. Further, there is only a small difference in the median level of suppressiveness of the 4545 s[rho]^{-} isolates when crossed
Variations in suppressiveness of spontaneous $\rho^-$ isolates from strain 4545 when picked at different times after plating out and crossed to two opposite wild-type strains.

The histograms correspond to:

(A, B) $s[\rho^-]$ picked at 63 hours, with a $[\rho]^+$ contamination rate of 38%, crossed to strain 4342 (A) (median level of suppressiveness 45%) and strain Ure 3.R$_2$ (B) (median level of suppressiveness 20%).

(P, Q) $s[\rho^-]$ picked at 111 hours, with a $[\rho]^+$ contamination rate of 0%, crossed to strain 4342 (P) (median level of suppressiveness 35%) and strain Ure 3.R$_2$ (Q) (median level of suppressiveness 25%).

The degree of suppressiveness is plotted to the nearest 5%. The arrows represent the same petite crossed to two opposite strains.
to strain 4342 at 63 and 111 hours (from 45-35%) despite a drop in \([\text{rho}]^+\) contamination of the putative \([\text{rho}^-]\) isolates from 40% to 0%. When crossed to strain Ure 3.R\(_2\) over the same period, there is no significant difference in the median level of suppressiveness (20% and 23%). Finally, as previously established, individual petites have a higher degree of suppressiveness when crossed to 4342 than when the same petite is crossed to Ure 3.R\(_2\).

**Discussion**

The results presented in this Chapter reflect on two inter-related properties concerning the mt DNA of *S. cerevisiae*. The first is the rate of breakdown of mt DNA to produce petites and the second is the suppressiveness patterns of petites from the same strain.

In relation to the first property, the buoyant density of the mt DNA of recently-arisen, spontaneous petites of *S. cerevisiae* does not differ significantly from the wild-type \([\text{rho}]^+\) DNA, despite a wide variation in the degree of suppressiveness both within a single strain and between different strains. This relationship holds for all strains and agrees with other results reported for three spontaneous petites from a single *S. cerevisiae* strain (Michels *et al.* 1974). However, in the present study it was demonstrated that the amount of mt DNA relative to that of main-band DNA is severely reduced in petites from strain 410. Such a drop in mt DNA content was not detected in petites from the other strains.
In the case of induced petites, where it is known that EtBr leads to big deletions from the mt DNA (Locker et al. 1974), it appears that a change in the buoyant density of mt DNA can be accompanied by a change in suppressiveness of the petite (Borst 1972), although no direct correlation exists between the two parameters (Michaelis et al. 1971). After severe EtBr treatment, the resulting petites lack all detectable mt DNA and have zero suppressiveness (Goldring et al. 1970; Nagley and Linnane 1970). On the other hand, in the study of faster-growing variants of spontaneous petites (Section 1-2) it was shown that continuous subculturing of petites leads either to a complete loss of mt DNA, or retention of mt DNA of unaltered buoyant density but of smaller, circular size. Thus in general, only when mutagens are used does a change in the buoyant density of the mt DNA result, despite the circular mt DNA being quite small in the spontaneous petites.

Differential rates of breakdown of the mt DNA of S. cerevisiae strains is shown firstly, by the [rho]+ contamination of the isolated putative respiratory-deficient mutants, and secondly, by the spontaneous petite frequency of the individual strains. In relation to the first indicator, the [rho]+ contamination of the morphologically identified petite colony, must arise from cells in the middle of the small colony, that have not, as yet, become [rho]−. Thus in such a mixed colony, those [rho]+ cells that have not yet become [rho]− are slower to decay than in other strains where no such [rho]+ cells are found in the putative respiratory-deficient isolate. It is observed that the percentage of [rho]+ contamination in the putative respiratory-
deficient isolates declines as the incubation time increases. In the case of the slowest decaying strain Ure 3.R₂, there is a 50% drop in the percentage of \([\text{rho}]^+\) contamination if the colonies are allowed to grow an extra 24 hours (48-72 hours) on the selective plates (Table 4-1).

The second property, that of spontaneous petite frequency, is felt to be an indicator of the excision rate of defective genomes from the \([\text{rho}]^+\) DNA. It is envisaged that a strain with a high spontaneous petite frequency must have a greater excision rate than a strain of low spontaneous petite frequency.

From these studies, evidence that the mt DNA of strain 410 (high spontaneous petite frequency) breaks down rapidly, is three-fold. Firstly, in most experiments, there is no \([\text{rho}]^+\) contamination of putative respiratory-deficient isolates when picked at the earliest possible time after plating, implying that the \([\text{rho}]^+\) DNA of these spontaneous petites has broken down very quickly. Secondly, the amount of mt DNA in these spontaneous petites is greatly reduced. Thirdly, from suppressiveness studies it is found that 410 s[\text{rho}]^- isolates show a near background level of suppressiveness when crossed to strain Ure 3.R₂. On the four occasions that the median level of suppressiveness of the 410 s[\text{rho}]^- is above the background level, it is found that the median level always falls to near the background in the next set of petites picked after a further 48 hours growth on GGYP plates. This situation is not shown by the other strains to nearly the same degree.
These points indicate that the mt DNA is breaking down to yield neutral petites at a more rapid rate in 410 compared to the other strains.

Supporting evidence for this conclusion with strain 410, comes from recent experiments on the induction of petites by EtBr (Wheelis et al. 1975). In this paper, it is shown by petite induction and recovery in the presence of EtBr, that the induced petites arising a short while after exposure to the drug show a certain level of reversion to the [rho]⁺ state. This level of reversion varies between strains, some being resistant to petite induction and showing high reversion to the [rho]⁺ state, whereas others are easily induced to form petites which exhibit no reversion to the [rho]⁺ state. Strain 410 was found to show no reversion to the [rho]⁺ state, reaching 100% petite formation after a few minutes exposure to the drug. Thus, strain 410 exhibited a rapid petite formation phase but failed to show any recovery to the [rho]⁺ state in the presence of EtBr, implying a rapid and irreversible loss of mt DNA from these cells.

Finally, a series of studies monitoring the loss or retention of antibiotic markers in petites have also demonstrated a differential breakdown of mt DNA between strains (Deutsch et al. 1973; Michels et al. 1974; Suda and Uchida 1974; Molloy et al. 1975).

The results of this Chapter also reflect on the suppressiveness patterns of spontaneous petites from the same strain. A direct correlation has been observed between the degree of suppressiveness of a group of petites and the
spontaneous *petite* frequency of the opposite wild-type strain. That is, a *petite* has a higher degree of suppressiveness when crossed to a strain of high spontaneous frequency, than the same *petite* has when crossed to another strain of lower *petite* frequency. This correlation holds for all the series of *petites* from the four strains, although occasionally an individual *petite* goes against the general pattern (e.g. one of the nine 4342 s[rho]−, Fig. 4-3A). This demonstration, that the degree of suppressiveness is not an absolute value has been reported previously for single *petites* crossed to two opposite mating-type strains, but the correlation between suppressiveness and the spontaneous *petite* frequency of the opposite strain has not been reported before (Michaelis *et al.* 1971; Waxman and Eaton 1974; Bech-Hansen and Rank 1973).

A further point of interest is revealed in crosses of wild-type and e[rho]° strains of 410 and Ure (Table 4-3), where the level of spontaneous *petite* frequency in the zygotes is high in all cases and resembles the *petite* frequency of 410 rather than Ure 3.R₂. Thus, in some manner, strain 410 has dominated this cross and imposed a high level of *petite* formation on the diploids. This dominance is not related to the presence or absence of mt DNA in strain 410 and hence would appear to be an influence of the nuclear background.

On the other hand, in a cross of the wild-type and e[rho]° strains of 410 and 4342, the level of *petite* frequency is low in all cases. Here, the level nearly equals the spontaneous *petite* frequency of 4342. In this case
strain 4342 has dominated the cross, imposing a low level of petite formation on the diploids, and again this dominance would appear to be a function of the nuclear background.

In the third situation between strain 4545 and Ure 3.R₂, it can be seen that the level of petite frequency is again higher than the spontaneous petite frequency of strain Ure 3.R₂ and nearly equals the petite frequency of 4545. Hence it would appear that 4545 dominates this cross.

Lastly, in crosses between strain 4545 and 4342, the spontaneous petite frequencies of both strains are similar, but it is possible that once again 4342 is dominant and the level of petite frequency is slightly lower than the spontaneous petite frequency of strain 4545.

Thus it would seem that the nuclear background of the strains, in some way influences a cross so that strain Ure 3.R₂ for instance, is dominated by both strains 410 and 4545, but strains 410 and 4545 are in turn dominated by strain 4342.

The interpretation of the result showing a relationship between suppressiveness and spontaneous petite frequency, is facilitated if it is accepted that the petite frequency is a measure of the rate of excision from mt DNA and that this is in turn caused by a nuclear gene product as discussed above. In the strains used, there is a hundred-fold difference in petite frequency between 410 and Ure 3.R₂, so that a strain showing a high petite frequency may have greater levels or higher specific activity of enzymes involved in excision events. It is then possible that these strains
cause a higher degree of suppressiveness due to an increased excision rate from the hybrid, partially duplicated molecules hypothesised to be intermediates in the suppressiveness process (Clark-Walker and Miklos 1974B). Of relevance to this idea is the fact that the spontaneous petite frequency appears to be under nuclear control, as strains isogenic for mt DNA but differing in nuclear background have different spontaneous petite frequencies (Chanet et al. 1973; Clark-Walker and Miklos 1975A). This nuclear background influence in spontaneous petite frequency directly correlates with the influence of the nuclear background over the level of suppressiveness reported in this study.

The notion is then formulated that nuclear factors could be involved in the excision rate of defective genomes from partially duplicated, hybrid molecules postulated to be formed in a suppressive cross. This idea could bear on both the recent report of the involvement of nuclear factors in suppressiveness (Waxman and Eaton 1974), and on the anomalous situation reported, where petites lacking any detectable mt DNA were found to be neutral when crossed to one [rho]⁺ strain, but suppressive when crossed to another (Bech-Hansen and Rank 1973).

Another factor that may be of importance in suppressiveness is the actual structure of the mt DNA of the S. cerevisiae strains. A recent study (Bernardi et al. 1975) has shown that the mt DNAs of wild-type strains differ in their number and location of endonuclease scission sites. Further the molecular weight estimates
from the banding patterns also show that the wild-type mt DNAs differ in size. Hence a contributing factor to the high spontaneous petite frequency of some strains may be the presence of more internal sites of partial homology in the respective mt DNA as compared to strains of low spontaneous petite frequency. The observed relationship between spontaneous petite frequency and level of suppressiveness may then be influenced by two factors, the previously discussed nuclear genes and the degree of insertion site homology. Thus if some mitochondrial DNAs have more homology than others, the frequency of insertion may be higher and this in turn may lead to increased suppressiveness.

That the degree of insertion between the [rho]− and [rho]+ genomes could be important in suppressiveness may be inferred from the study of the low buoyant density petite RD1A. This petite has much altered mt DNA yet shows zero suppressiveness (Moustacchi 1972). The structure of RD1A mt DNA is a repeating unit of a 70 nucleotide-long base sequence, amplified many times, which occurs in the cell in the form of 'fishnet concatenanes' (Hollenberg et al. 1972). This mt DNA structure would mean that there is probably very little homology with [rho]+ genomes, hence there would be very little insertion taking place in a suppressiveness cross.

It would appear then that the degree of suppressiveness of a petite strain is probably the result of at least two factors, these being nuclear genes coding for enzymes
involved in the insertion and excision processes and secondly, the particular structures of the mitochondrial DNAs involved.
General discussion

The work described in the present study concerns phenomena associated with the petite mutation in yeast. Two areas were chosen for investigation based on ideas formulated by Clark-Walker and Miklos (1974B) in their general discussion of the mechanism of the petite mutation. The areas chosen for study being the phenomenon of petite-negative yeasts and the process of suppressiveness. Furthermore, petite negativity has been approached in two ways, namely structural studies on mt DNA and secondly, by investigating the slow growth phenomenon of petite mutants.

Two sections of this study (Chapters 2 and 3) have been concerned with investigating the structure of mt DNA. The first section deals with mt DNA from petite-negative yeasts and the second, mt DNA from petite-positive yeasts.

It has been shown that the mt DNA from petite-negative yeasts varies in size between 6-12 μm, as compared to the 25 μm mt DNA of the petite-positive yeasts S. cerevisiae and S. carlsbergensis. The mt DNA of most petite-negative yeasts studied was found to be higher in buoyant density than the mt DNA of S. cerevisiae, and only one yeast, H. wingei, had mt DNA that was of similar buoyant density (1.686 g/cm³). It has also been demonstrated that petite-negativity is not related to the presence or absence of other species of extrachromosomal DNA. Thus S. rouxii, a petite-negative yeast, was found to have small, circular DNA closely resembling, in both size and buoyant density, the omicron DNA of S. cerevisiae.
In initial studies, the closed-circular DNA bands of the petite-negative yeasts *H. wingei* and *C. parapsilosis* were found to contain a large proportion of small, circular DNA molecules. By purification of mitochondria from *H. wingei* on a sucrose gradient, it was shown that both the small, circular DNA and the minor peak of main-band DNA buoyant density could be eliminated entirely from the closed-circular DNA band. The resulting closed-circular DNA contained a single species of length 8 \( \mu \)m and buoyant density 1.686 g/cm\(^3\), thereby showing that the small DNA circles are not excision products of the mitochondrial genome. Nevertheless, there are reports in the literature that some other petite-negative yeasts, like *H. wingei*, possess mt DNA rich in A + T (Luha *et al*. 1971, 1974), and further, that some of these mt DNAs, like that from *S. cerevisiae*, show discrepancies in the G + C content as determined from Tm and buoyant density data (Bak *et al*. 1969). These observations and the results of this study have been collated into a single table (Table 5-1).

In the case of these petite-negative yeasts that possess mt DNA rich in A + T, the data might mean that their mt DNA has similar structural features to *S. cerevisiae* mt DNA and may therefore be capable of undergoing excision events. If this is the case, then other explanations need to be sought for petite-negativity. However, some yeasts have mt DNA with a much higher G + C content. These yeasts may not have the AT-rich regions associated with *S. cerevisiae* mt DNA. In these cases, the postulate still holds that
## TABLE 5-1

Yeast mt DNA data

<table>
<thead>
<tr>
<th>Species</th>
<th>Buoyant density g/cm³</th>
<th>% G + C</th>
<th>Tm °C</th>
<th>% G + C</th>
<th>Chem. analysis % G + C</th>
<th>Length (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. cerevisiae</td>
<td>1.684</td>
<td>24.5</td>
<td>74.5</td>
<td>12.7</td>
<td>21</td>
<td>21-25</td>
</tr>
<tr>
<td>S. carlsbergensis</td>
<td>1.684</td>
<td>24.5</td>
<td></td>
<td></td>
<td></td>
<td>25</td>
</tr>
<tr>
<td>T. glabrata</td>
<td>1.686</td>
<td>26.5</td>
<td></td>
<td></td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>Br. anomalus</td>
<td>1.684</td>
<td>24.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D. intermedia</td>
<td>1.685</td>
<td>25.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D. bruxellensis</td>
<td>1.686</td>
<td>26.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kl. africana</td>
<td>1.686</td>
<td>26.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Petite-ve**

<table>
<thead>
<tr>
<th>Species</th>
<th>Buoyant density g/cm³</th>
<th>% G + C</th>
<th>Tm °C</th>
<th>% G + C</th>
<th>Chem. analysis % G + C</th>
<th>Length (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. lipolytica</td>
<td>1.687</td>
<td>27.6</td>
<td>78.0</td>
<td>21.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. pelliculosa</td>
<td>1.688</td>
<td>28.6</td>
<td>76.5</td>
<td>17.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. pseudotropicalis</td>
<td>1.684</td>
<td>24.5</td>
<td>75.0</td>
<td>13.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. utilis</td>
<td>1.685</td>
<td>25.5</td>
<td>77.0</td>
<td>18.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. zeylanoides</td>
<td>1.692</td>
<td>32.7</td>
<td>80.0</td>
<td>26.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. parapsilosis</td>
<td>1.698</td>
<td>38.8</td>
<td></td>
<td></td>
<td></td>
<td>11</td>
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<tr>
<td>K. lactis</td>
<td>1.692</td>
<td>32.7</td>
<td>82.7</td>
<td>32.7</td>
<td></td>
<td>11</td>
</tr>
<tr>
<td>K. fragilis</td>
<td>1.683</td>
<td>23.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H. wingei</td>
<td>1.686</td>
<td>26.5</td>
<td></td>
<td></td>
<td></td>
<td>8</td>
</tr>
<tr>
<td>S. pombe</td>
<td>1.695</td>
<td>35.7</td>
<td></td>
<td></td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>S. rouxii</td>
<td>1.692</td>
<td>32.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. rosei</td>
<td>1.693</td>
<td>33.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data from: Bernardi *et al.* 1970  
Luha *et al.* 1971, 1974  
Bak *et al.* 1969  
Smith *et al.* 1968  
This thesis
structural properties of mt DNA may be important in determining petite-negativity.

In view of the results obtained with the mt DNAs of petite-negative yeasts, the study was widened to encompass petite-positive yeasts other than in the genus *Saccharomyces*. Compilation of data in the literature showed that two ascomycetous species and three asporogenous species, unrelated to *Saccharomyces*, were petite-positive and deserved further study. It was found that all five species had firstly, mt DNA of low buoyant density and secondly, a low frequency of spontaneous respiratory-deficient mutants. A detailed analysis of one species, *Torulopsis glabrata*, showed that this petite-positive yeast had mt DNA of 6 μm in length and having a buoyant density of 1.686 g/cm³. The mt DNA of the wild-type could be eliminated by EtBr treatment, resulting in respiratory-deficient mutants lacking all detectable mt DNA. Analysis of spontaneously-arisen respiratory-deficient isolates of *T. glabrata* showed that, although they arise at a very low frequency, all had mt DNA of altered buoyant density. This change in buoyant density was accompanied by the appearance of smaller, circular DNA molecules, which is reminiscent of petites from *S. cerevisiae*. This result suggests that respiratory-deficient mutants of *T. glabrata* are formed by a similar mechanism to that yielding petites in *S. cerevisiae*

The main implication of this finding with *T. glabrata* is that there is not a simple relationship between genome size and the presence or absence of AT-rich sequences as was originally postulated for petite-negative yeasts (O'Connor
et al. 1975). These results are also another indication that simple structural differences between mt DNAs may not be of prime importance in determining petite-negativity and hence it appears that other factors must also be involved.

Recently, evidence has come from bacteriophage studies implicating the regions of high A + T as the site of the excision-insertion processes postulated to occur in yeast mt DNA. Reports have indicated that most non-essential gene deletions from the bacteriophage genomes of λ and P₂ map in the AT-rich regions of the molecules. In the case of P₂ DNA, a total of five deletions occur in the relatively A + T rich segments of the genome (Chattoraj et al. 1975), whilst with λ DNA the deletion mutations map in three regions of the genome, all of which are in the right, A + T richer half of the DNA (Davidson and Szybalski 1971). However, the present results indicate that it now seems highly unlikely that structural differences between mt DNAs are the primary cause of petite-negativity and hence other factors have to be considered.

One of the other factors postulated to be involved in petite-negativity is the presence on the mt DNA of a viability gene or genes (Clark-Walker and Miklos 1974B). In this respect, the slow growth phenomenon of petites of S. cerevisiae was studied, in order to ascertain if this slow growth phenomenon concerned a mt DNA encoded gene contributing to viability. The studies reported herein have shown that all petites grow slower than the parent wild-type strain in a glucose medium. Further, a nuclear respiratory-
deficient mutant was also shown to have a reduced growth rate compared to the wild-type strain, despite the fact that it retained a complete mitochondrial genome which is most probably transcribed. Hence, this finding with the nuclear respiratory-deficient mutant would seem to negate the postulate of Clark-Walker and Miklos (1974B) that a mitochondrial transcription product, translated in the cytoplasm, is necessary for optimal cell growth. Nevertheless the interpretation is complicated because there exists another group of nuclear respiratory-deficient mutants, associated with the mitochondrial translocase systems, for which the presence of intact mt DNA is crucial, so that loss or alteration of the mt DNA by petite induction leads to cell arrest and death.

Consideration of this growth data has resulted in a re-interpretation of the viability gene postulate for mt DNA. It is now envisaged that in *S. cerevisiae* the coding for the vital function or functions that the mitochondrion provides in the cell, is divided between the nuclear and mitochondrial genomes. Loss of one set of functions results in some cell growth inhibition, but loss of both sets of functions results in cell arrest and death. In the case of petite-negative yeasts it is envisaged that the coding for these vital functions is all on the mt DNA, so that loss of intact mt DNA leads to loss of some of these vital functions and hence cell death.

A further factor that has to be considered in petite-negativity is the enzymes involved in the excision-insertion
processes. It is well documented that the spontaneous petite frequency varies between different strains of *S. cerevisiae*, and in the studies reported here there is a hundred-fold difference in petite frequency between strains 410 and Ure 3.R₂. Additionally, the other petite-positive yeasts outside the genus *Saccharomyces* were also found to have low frequencies of spontaneous respiratory-deficient mutants. Hence, the question arises as to whether this low petite frequency found with Ure 3.R₂ and these other petite-positive yeasts is due to either a basic difference in the structure of their mt DNA, or whether it is due to a low level of excision enzymes in these strains. If the latter proves to be the case, then the extrapolation of this idea is that petite-negative yeasts may lack such excision enzymes.

In relation to these excision-insertion enzymes, it appears that these enzymes are under nuclear control (Chanet *et al.* 1973, Clark-Walker and Miklos 1975A). Additionally, the results of this study on suppressiveness, show that there is a direct correlation between spontaneous petite frequency of a wild-type strain and the level of suppressiveness of petites that are crossed to this particular strain. For instance, a strain showing a high frequency of petites causes a higher level of suppressiveness in petites with which it is crossed. Further, there appears to be a form of nuclear-controlled dominance operating in the suppressiveness crosses. It seems likely that these results are related in some manner to the enzymes involved in excision-insertion events, but they could possibly be due to the actual structure of the different mt DNAs. Thus it is apparent that more than
one factor contributes to the phenomenon of suppressiveness. It is also felt that petite-negativity may have multiple factors. Firstly, a difference in mt DNA structure may still be involved in those yeasts with mt DNA of high G + C content. However, this factor is probably not involved in all cases. Secondly, petite-negativity could be due to the presence of viability genes on the mt DNA of these yeasts. The importance of intact mt DNA is emphasised by the op1, pet936 and Z1 x 1 group of nuclear respiratory-deficient mutants of S. cerevisiae, and hence in petite-negative yeasts intact mt DNA may be even more important, in fact vital. Thirdly, the enzymes necessary for the excision-insertion processes may be absent or inactivated in petite-negative yeasts where the spontaneous petite frequency is zero.
APPENDIX I

MATERIALS AND METHODS
(A) Materials

(i) Yeast strains

The strains of *S. cerevisiae* and other yeast species are listed in Tables IA and IB respectively.

(ii) Chemicals

Chloramphenicol was a generous gift of Parke, Davis and Co., Caringbah, N.S.W. 2229; Ethidium bromide (2, 7-Diamino-10-ethyl-9-phenylphenanthridium bromide) was obtained from Calbiochem, San Diego, California, 92112; Caesium chloride (Sequanol Grade) was obtained from Pierce, Box 117, Rockford, Illinois, 61105, U.S.A. and caesium chloride (AR grade) from Ajax Chemicals Ltd., Sydney. All other reagents are AR grade. Snail juice (Suc d'Helix Pomatia-stabilisé standardisé) was obtained from Industrie Biologique Francaise, Gennevilliers 92231, France.

(B) Methods

(i) Media

(a) Complete media

The basic yeast extract, peptone medium (YP) contains per litre: 3 gm KH$_2$PO$_4$, 5 gms yeast extract (Difco), 10 gms bactopeptone (Difco). To this is added for: 1% GYP medium, 10 gms of glucose; for 2% GYP medium, 20 gms of glucose; for Gly YP medium, 40 gms glycerol; for EtYP medium, 40 mls of 96% ethanol and for GGYP medium, 40 gms glycerol and 2 gms glucose. Media are solidified by the addition of 15 gms per litre agar (Difco).
**TABLE IA**

**STRAINS OF SACCHAROMYCES CEREVISIAE USED**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Haploid (H) or Diploid (D)</th>
<th>Mating Character</th>
<th>Auxotrophic markers</th>
<th>Requirements</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>4342</td>
<td>H</td>
<td>α</td>
<td>lys&lt;sup&gt;−&lt;/sup&gt;, ade&lt;sup&gt;−&lt;/sup&gt; 8-18</td>
<td>lysine, adenine</td>
<td>S. Fogel, Department of Genetics, Berkeley, USA.</td>
</tr>
<tr>
<td>4545</td>
<td>H</td>
<td>α</td>
<td>arg&lt;sup&gt;−&lt;/sup&gt; 4-16, ade&lt;sub&gt;1&lt;/sub&gt;</td>
<td>arginine, adenine</td>
<td></td>
</tr>
<tr>
<td>4545 ERY&lt;sup&gt;R&lt;/sup&gt;</td>
<td>H</td>
<td>α</td>
<td>arg&lt;sup&gt;−&lt;/sup&gt; 4-16, ade&lt;sub&gt;1&lt;/sub&gt;</td>
<td>arginine, adenine</td>
<td>Spontaneously arising erythromycin-resistant mutant from previous strain.</td>
</tr>
<tr>
<td>410</td>
<td>H</td>
<td>α</td>
<td>his&lt;sup&gt;−&lt;/sup&gt;</td>
<td>histidine</td>
<td>From strain 41 of D. Wilkie, University College, London.</td>
</tr>
<tr>
<td>Ure 3.R&lt;sub&gt;2&lt;/sub&gt;</td>
<td>H</td>
<td>α</td>
<td>ade&lt;sub&gt;2&lt;/sub&gt;</td>
<td>adenine</td>
<td>A red adenine requiring mutant from strain MA&lt;sub&gt;1&lt;/sub&gt;-sp&lt;sub&gt;1&lt;/sub&gt; (URE-3) of M. Aigle, Strasbourg, France.</td>
</tr>
<tr>
<td>50 Rα</td>
<td>H</td>
<td>α</td>
<td>ade&lt;sub&gt;1&lt;/sub&gt;, arg&lt;sup&gt;−&lt;/sup&gt; 4-16</td>
<td>adenine, arginine</td>
<td>Segregant from two other strains of G.D. Clark-Walker: 4342 ERY&lt;sup&gt;R&lt;/sup&gt; CAP&lt;sup&gt;R&lt;/sup&gt; x 4545e [rho]&lt;sup&gt;−&lt;/sup&gt;.</td>
</tr>
<tr>
<td>M</td>
<td>D</td>
<td>−</td>
<td></td>
<td></td>
<td>A. W. Linnane, Dept. of Biochemistry, Monash University, Melbourne</td>
</tr>
</tbody>
</table>
### TABLE IB
OTHER YEAST SPECIES USED

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Auxotrophic markers</th>
<th>Requirements</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>(1) Petite-negative</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Candida parapsilosis</td>
<td></td>
<td>-</td>
<td>-</td>
<td>A. W. Linnane, Department of Biochemistry, Monash University, Melbourne.</td>
</tr>
<tr>
<td>Kluveromyces lactis</td>
<td>W231B</td>
<td>his&lt;sup&gt;-&lt;/sup&gt;</td>
<td>histidine</td>
<td>A. Herman, Northern Region Research Laboratories, Peoria, Illinois, USA.</td>
</tr>
<tr>
<td>Hansenula wingei</td>
<td>V31B</td>
<td>met&lt;sup&gt;-&lt;/sup&gt;</td>
<td>methionine</td>
<td></td>
</tr>
<tr>
<td>Saccharomyces rouxii</td>
<td>C.B.S. 732</td>
<td>-</td>
<td>-</td>
<td>Centraalbureau voor Schimmelcultures Yeast Division, Delft, The Netherlands.</td>
</tr>
<tr>
<td>Saccharomyces rosei</td>
<td>C.B.S. 817</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Schisosaccharomyces pombe</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>E. H. Creaser, Research School of Biological Sciences, ANU, Canberra.</td>
</tr>
<tr>
<td><strong>(2) Petite-positive</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brettanomyces claussenii</td>
<td>C.B.S. 76</td>
<td>-</td>
<td>-</td>
<td>Centraalbureau voor Schimmelcultures Yeast Division, Delft, The Netherlands.</td>
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<tr>
<td>Brettanomyces anomalus</td>
<td>C.B.S. 77</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Dekkera bruxellensis</td>
<td>C.B.S. 74</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Dekkera intermedia</td>
<td>C.B.S. 4914</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Kloechera africana</td>
<td>C.B.S. 277</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Torulopsis glabrata</td>
<td>C.B.S. 138</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>
(b) Synthetic media

The basic synthetic medium (SyVit) contains per litre:

- 0.5 gm KH$_2$PO$_4$
- 1.5 gm (NH$_4$)$_2$SO$_4$
- 0.1 gm NaCl
- 0.1 gm CaCl$_2$
- 2H$_2$O
- 0.5 gm MgSO$_4$.7H$_2$O
- 5.4 mg ferric citrate .3H$_2$O
- 5 mls of a trace-metal solution containing per 5 ml:
  - 0.2 mg CuSO$_4$.5H$_2$O
  - 0.5 mg KI
  - 0.5 mg ZnSO$_4$.7H$_2$O
  - 0.5 mg Na$_2$Mo.O$_4$
- 2H$_2$O
- 0.5 gm Na$_2$B$_4$O$_7$.10H$_2$O
- 1.0 mg MnSO$_4$.4H$_2$O
- 5 mls of a freshly prepared vitamin mixture containing per 5 ml,
  - 1 mg calcium pantothenate
  - 1 mg thiamine
  - 1 mg pyridoxine
  - HCl
  - 2 mg inositol
  - 0.5 mg nicotinic acid
  - 0.2 mg biotin

To this is added for Glu SyVit medium, 20 gms glucose; for
Gly SyVit medium, 40 gms glycerol, and for GG SyVit medium
40 gms glycerol and 2 gms glucose. Media are solidified
by the addition of 15 gms per litre agar (Difco).

(ii) Ethidium-bromide generated petite colonies

To ensure the isolation of colonies that had completely lost mitochondrial DNA of buoyant density 1.684 g/cm$^3$, yeast cells were exposed to ethidium bromide at the highest concentration compatible with growth. A drop of ethidium bromide solution (10 mgs/ml) was placed 1-2 cms from the perimeter of an agar plate containing GGYP medium. After the drop had diffused into the agar, a loop-full of yeast culture was streaked across the dye and the plate incubated at 30°C. After 24-48 hrs incubation, cells from the margin of growth near the dye were streaked away from the ethidium bromide (photo I-1) and single colonies were picked after a further 48 hrs of incubation. Respiratory-deficient, [rho]$^-$ colonies, which are small, can easily be
PHOTO I-1

Production of ethidium-bromide generated petite colonies

The photograph shows a GGYP plate with a drop of EtBr (10 mgs/ml) dried into the agar and a yeast culture streaked through the drop. The culture is then sub-streaked from the margin of growth on the plate. After incubation, individual colonies are picked and tested for respiratory-deficiency. The strain used in the photo is S. cerevisiae strain M.
distinguished from larger \([\text{rho}]^+\) colonies on this medium.

(iii) **Spontaneous petite colonies**

Yeast cells were grown for 24 hrs in EtYP liquid medium, plated on GGYP medium and putative respiratory deficient colonies were selected by their small size after 3 days growth at 30°C. The putative mutants were resuspended in 0.5 ml sterile water and aliquots dropped onto GYP and Gly YP plates. After 48 hrs growth the plates were scored and those putative mutant colonies that grew on GYP plates but failed to grow on Gly YP were kept as true petite-colonies.

(iv) **Culture conditions**

Yeast cultures of 1 litre, contained in 3-litre conical flasks, were inoculated with 5-8 mg (dry weight) of exponentially growing cells and incubated at 30°C overnight on a gyrorotary shaker operated at 200 r.p.m.

(v) **Fractionation of yeast**

Cultures that had reached the late exponential to early stationary phase of growth, (approx. 2-5 mg dry weight/ml) were harvested by centrifugation for 5 mins at 1500 x g and resuspended in cold buffer (ESE) containing 0.5M sorbitol,0.01 M EDTA and 0.5 gms/litre EtBr, adjusted to pH 7.0, followed by centrifugation as above. Cells were broken with 0.5 mm glass beads by shaking for 25 secs in a Braun homogenizer (B. Braun, Melsungen, W. Germany) which was cooled by expansion of \(\text{CO}_2\). Unbroken cells, cell-wall debris and nuclei were removed by two centrifugations at 2000 x g for 5 mins and the mitochondrial fraction collected by centrifugation at 8500 x g for 20 mins. The
pellet from this centrifugation was resuspended in the above ESE buffer, and sedimented as before. All operations were done at 0-4°C under dim light to minimize damage to the DNA by photoactivated dye.

(vi) Sucrose gradient centrifugation

Linear gradients containing 34 mls of 25-60% (w/v) sucrose in 0.01 M EDTA pH 7.0, 0.01 M Tris HCl (pH 7.4) (that is: 25 gms of sucrose + 75 mls of solvent and 60 gms of sucrose + 40 mls of solvent) were overlayed with 2 mls of crude mitochondria-enriched suspension. The gradients were centrifuged for 1 hr at 10°C in a Beckman SW27 rotor operated at 25,000 r.p.m. (81,000xg max). Particulate bands were collected by side puncture of the tubes with an 18-gauge needle and withdrawal of the band with a syringe. The fractions were then diluted to 20 mls in ESE buffer, and the particles collected by sedimentation at 10,000 x g for 20 mins.

(vii) Spheroplast preparation

Cells to be converted to spheroplasts were suspended in buffer 1 (0.1M Tris-HCl pH 9.3, 0.02M EDTA, 0.1M mercaptoethanol), for 10 mins at room temperature, centrifuged at 1500 x g and resuspended and washed twice in buffer 2 (0.9M sorbitol, 0.02M imidazole-HCl pH 6.4, 1 mM EDTA) at room temperature. Snail digestive juice (from Industrie Biologique Francaise) comprising one fifth the final volume, was added and the suspension kept at 33°C in a water bath. Spheroplast formation was monitored by following the decrease in absorption of 1000-fold diluted samples at 640 nm. When the absorbance had fallen to 10% of the initial value, the suspension was diluted 1 in 4
with chilled buffer 2, centrifuged at 1000 x g for 5 mins, and the spheroplasts washed twice in the same buffer. The pellet was resuspended in 2 volumes of buffer 2 and disrupted by passage through a large French pressure cell operated at 2500 psi, diluted 1:4 with ESE buffer and, after removal of unbroken cells and debris by two centrifugations at 2500 x g, a crude mitochondrial pellet was obtained by centrifugation at 8,500 x g for 20 min.

(viii) Dye-buoyant density centrifugation

The DNA in the crude mitochondrial pellet obtained either from the yeast fractionation or the sucrose gradient centrifugation, was released into solution by resuspension of the pellet by gentle agitation in 1 ml of 2% Sarkosyl for 5 mins at 0-4°C under a red safety light. Dye-buoyant density centrifugation was performed in polyallomer tubes in either a Beckman 50 Ti or 60 Ti rotor. The former tubes, 3" x 5/8" contained 4.3 gms CsCl, together with 1.4 mls of ethidium bromide solution (1.75 mg/ml in 0.1 M sodium phosphate buffer (pH 7.0)), 0.3 ml of 0.1 M EDTA (pH 7.0) and 2.8 mls of the resuspended pellet and water to bring the total volume to 4.5 mls. For the 60 Ti rotor, the 3.5" x 1" tubes contained 14 gms CsCl, together with 4 mls of ethidium bromide solution, 1 ml of EDTA and 9 mls of resuspended pellet and water, to bring the total volume added to 14 mls. The solution was overlayed with liquid paraffin and centrifuged at 10°C in either a Beckman 50 or 60 titanium rotor at 48,000 rpm for 48-72 hrs. After centrifugation, the DNA bands were visualised by ultraviolet light (λ = 350 nm) excited fluorescence of the intercalated
ethidium bromide and the DNA removed by side-puncture of the tube using an 18-gauge needle.

(ix) **Analytical ultracentrifugation**

DNA fractions were prepared for analytical ultracentrifugation by removal of the bound ethidium bromide by four extractions with isoamyl alcohol. The remaining isoamyl alcohol and CsCl were removed by overnight dialysis against $\frac{1}{10}$ SSC (SSC contains 10 mM EDTA - 15 mM NaCl - 1.5 mM sodium citrate (pH 7.0)). Buoyant density was determined in either a Beckman Model E ultracentrifuge with an AnF-titanium rotor and double-sector, charcoal-filled cells or in an MSE centriscan with an AN-6, six-hole analytical rotor and single-sector, epoxy-resin centrepiece cells. About 2 μg of DNA, together with an equal quantity of *Micrococcus luteus* DNA, was added to an optical-grade CsCl solution and the density adjusted to 1.710 g/cm³ at 25°C with the aid of a refractometer. To make up DNA for running in the ultracentrifuge: the blank consists of 645 mgs optical grade CsCl + 500 mgs 0.01 M Tris/HCl pH 7.4. The refractive index at 25°C should be between 1.400 and 1.401. DNA samples must have 0.01-0.03 optical density units per sample, that is if the DNA solution has an O.D. 260 nm of 1.0, then add 0.01 ml. If a marker DNA is to be added, then add 0.01 O.D. units of the marker also. The centrifuge was operated at 44,000 rpm at 20°C (MSE) or 25°C (Model E) and the DNA bands visualized after 20-24 hrs with a Multiplex photoelectric scanning system operated at a wavelength of 264 nm (Model E) or 254 nm (Centriscan).
Buoyant densities were calculated from traces (Mandel et al. 1968) on the basis of a buoyant density of 1.731 g/cm³ for the *M. luteus* marker using the approximating equation (Sueoka 1961): 

$$\rho = \rho_0 + 4.2\omega^2 (r^2 - r_0^2) \times 10^{-10} \text{ g/cm}^3,$$

in the region of density from 1.65 - 1.75 g/cm³.

(x) **Electron microscopy**

Visualization of DNA was by the protein film technique (Kleinschmidt and Zahn 1959) as modified by Davis et al. (1971). A solution of DNA for spreading was prepared by mixing 25 μl 5M ammonium acetate, 25 μl cytochrome C (1 mg/ml), 25 μl DNA (10 μg/ml) and 175 μl double distilled, double deionized water. This solution (50 μl) was spread onto a clean hypophase of 0.25M ammonium acetate in a Teflon-coated aluminium trough. Areas of the surface were picked up on freshly prepared parlodion films supported on 400-mesh copper grids, stained by immersion in 0.05 mM uranyl acetate in 90% ethanol for 30 seconds and excess stain removed by immersion in redistilled isopentane for 10 seconds. Photographs were taken at a magnification of 9,800 using a Hitachi HuIIe electron microscope operated at 75 Kv or at a magnification of 11,200 using a JEM 100c electron microscope operated at 80 Kv. For molecular weight measurements, the circular yeast DNA was spread with circularised λcI 857 DNA, which was a generous gift of Dr. A. Bellett, John Curtin School of Medical Research, The Australian National University. The molecular weight of λ DNA was taken to be 30.8 x 10⁶ (Davidson and Szybalski 1971). Lengths of relaxed circular molecules were measured with a map measurer, after projection of the negatives onto
a screen giving an additional magnification of 17.3 times.

(xi) Preparation of DNA from *Micrococcus luteus*

(a) Growth medium

Meat infusion broth. For 1 litre of medium add:
- 10 gms Difco Heart infusion or beef extract, 10 gms Difco protease peptone, 3 gms Difco yeast extract, 5 gm sodium chloride, 5 gms glucose. 1 litre of medium in a 3 litre flask is inoculated with a loop of bacteria from a slope, and shaken at 200 r.p.m. for 24 hrs at 30°C. The culture is harvested at the end of exponential growth, at an optical density at 540 nm of approximately 5.0, by centrifugation for 5' at 5000 xg and washed twice by resuspension in 'saline-EDTA' (0.15M NaCl, 0.1M EDTA pH 8.0) followed by centrifugation as above. The washed cells are suspended in saline-EDTA to give a total volume of 50 mls.

(b) Preparation of DNA

The cell suspension is digested with lysozyme at a final concentration of 1 mg/ml, for 60' at 30° and lysed by the addition of 0.35 ml of 30% sarkosyl, to give a final concentration of 0.2% sarkosyl. Pronase (50 mgs in 5 ml of 0.1M Na phosphate pH 7.0, self-digested at 30°C for 5 hours) is added to give a final concentration of 1 mg/ml, and the solution incubated at 30°C overnight.

The pronase digested solution is distributed into glass, stoppered, centrifuge tubes of total capacity 15 ml; 8 ml being added to each tube. Phenol 5 ml (showing no pink colouration) and saturated with SSC is added to each tube, the stopper taped on and the tube gently agitated by rotation for 20' at room temperature. (The phenol is
prepared by melting solid phenol and equilibrating this liquid with SSC (0.15M NaCl, 0.015 M Tri Na Citrate pH 7.0) in a separating funnel.

The phenol and aqueous phase are separated by centrifugation at 3,000 r.p.m., for 10 mins in an MSE bench centrifuge after removal of the stoppers. The upper aqueous phase containing the DNA is withdrawn from the lower phenol phase and interface which contains denatured protein. The phenol extraction is repeated three times and the DNA precipitated by the slow addition of an equal volume of absolute ethanol. The DNA is removed by spooling onto a glass rod, washed with ethanol and redissolved in 10 ml of \( \frac{1}{10} \) dilute SSC by allowing to stand overnight at 2°C.

As an indication of DNA purity at this stage, the absorbance of a sample is measured at 260 nm and 280 nm. The ratio of 260/280 absorbance should be around 1.9 for pure DNA. If protein is still present, the ratio will be lower and the phenol extraction step is repeated. If the DNA is required free of RNA, the sample is digested with pancreatic ribonuclease by incubating at 30°C for 60 min with a final nuclease concentration of 50 µg/ml. The RNAase is pretreated to remove residual DNAase by heating a 1 mg/ml solution in 0.1N Na phosphate pH 7.0 to 85°C for 10'.

The concentration of DNA can be measured at 260 nm using the value of an absorbance of 1.0 equivalent to a DNA concentration of 50 µg/ml.

(xii) Sporulation and ascus dissection

Cultures to be induced to sporulate were grown in GYP liquid medium to late exponential phase and then 0.3 ml was
spread on KAc agar (potassium acetate anhydrous .005%, agar 1.5%). After 4 days at room temperature (23°C) asci were dissected with a de Fonbrune pneumatic micromanipulator after brief treatment with snail digestive tract enzyme (Johnston and Mortimer 1959). Dissected ascospores were incubated for 3 days at 30°C, the samples were resuspended in water and then inoculated onto selective plates to determine nutritional requirements.

(xiii) **Determination of suppressiveness**

As originally stated, the degree of suppressiveness of a \( \rho \) strain is calculated from the percentage of zygotes giving rise to \( \rho \) diploid clones following a cross with a normal, wild-type strain (i.e. the percentage of \( \rho \) diploid colonies arising on the minimal medium plates) (Ephrussi et al. 1955). This concept was modified (Ephrussi and Granchamp 1966) to take into account the percentage of spontaneous \( \rho \) colonies in the parent wild-type strain from which the \( \rho \) isolate arose, and the degree of suppressiveness was expressed as:

\[
\% S = \frac{(X - Y) \times 100}{100 - Y}
\]

where \( X \) = percentage of \( \rho \) diploids on the minimal medium plates;

\( Y \) = percentage of spontaneous \( \rho \) colonies in the parent wild-type strain.

The degree of suppressiveness was redefined by Nagley et al. (1973), who corrected the estimations of suppressiveness for the background of spontaneous \( \rho \) colonies arising in the wild-type strains. This correction was made
by measuring the frequency of \( \text{[rho]}^- \) zygote clones when a reference strain of zero suppressiveness, derived from the same strain as the \( \text{[rho]}^- \) isolates, was crossed to the same wild-type strain of opposite mating type. If the minimal medium plates, after a test cross, yield \( M \) small colonies and \( L \) large colonies and the reference zero suppressiveness cross yields \( m \) small colonies and \( 1 \) large colonies, then the corrected suppressiveness of the test \( \text{[rho]}^- \) is given by:

\[
\frac{100 \times M}{L + M} - \frac{M}{L + M} = \frac{1}{1 + \frac{m}{L + M}}
\]

For this thesis, the latter formula was deemed unwieldy and the degree of suppressiveness is taken from the original relationship:

\[
\% S = \frac{\text{number of } \text{[rho]}^- \text{ diploids}}{\text{number of total diploids}} \times 100
\]

This degree of suppressiveness is expressed along with both the percentage of \( \text{[rho]}^- \) diploids arising from the cross of the two wild-type strains, and also the percentage of \( \text{[rho]}^- \) diploids arising from the cross of a neutral \( \text{[rho]}^- \) isolate, known to lack all detectable mt DNA, of the same parent crossed to the same wild-type strain of opposite mating type.

**Method**

The strains crossed together in the determination of suppressiveness must be complementary for auxotrophic nuclear markers, this ensures that only diploids will be able to grow on the minimal media plates. Hence it is best to use strains
with double auxotrophic markers to guard against reversion of single mutants.

The test \([\text{rho}]^-\) isolate was grown overnight in 5 ml of 2% GYP medium at 30°C. The wild-type strain was grown over a 48 hr period in 50 ml of 4% EtYP medium at 30°C. 1 ml of both the test \([\text{rho}]^-\) isolate and of the opposite wild-type strain were added to 3 ml of 3% GYP medium and the mixture shaken for 3 hours on a reciprocal shaker at 30°C. The mixture was then left to stand for another 3 hours at 30°C and then the suspension was disrupted vigorously with a vortex mixer for 3 minutes and serially diluted from \(10^{-1}\) to \(10^{-5}\). 0.1 ml aliquots of each dilution were plated in triplicate on GGSyVit plates and the plates incubated at 30°C for 4 days. After incubation, the number of small and large diploid colonies on the selective medium plates were scored.

(xiv) Growth rate determination

These were determined by following the changes in optical density at 640 nm of cultures growing in 50 ml of 1% or 2% GYP medium in 125 ml conical flasks shaken at 200 r.p.m. The cultures were maintained at 30°C in a gyro-rotary water bath shaker. Dry weight can be related to optical density using the values given in each section for the different yeast species used.

(xv) Method of repeated sub-culture

An initial culture was grown in 2% liquid GYP over-night at 30°C. The optical density at 640 nm of this primary culture was measured (a) and a known volume (y) normally 0.05 ml, was used to inoculate a second 125 ml conical
flask containing 50 mls of 2% GYP medium. This second culture was grown overnight at 30°C and both the final optical density at 640 nm (b) and the volume of the second culture (z) are measured. The number of generations (n) undergone by the culture is then given by the relationship:

\[ n + 1 = \frac{p}{q} \]

where \( p \) = initial mass of cells in culture

\( q \) = final mass of cells in culture

or

\[ (n + 1) \log p = \log q \]

which using the measured values, simplifies to:

\[ n + 1 = \log_{10} \frac{2}{\log_{10} 2} \cdot \frac{b}{z} \]

(xvi) Renaturation kinetic analysis

Isolated mitochondrial DNA was sheared to an \( S_{20w}^{B_{13}} \) of 6.05 by passage through a French pressure cell at 15,000 psi and then dialysed for 24 hr against two changes of 1M NaCl. The DNA was denatured in a Teflon stoppered quartz cuvette for 5 min in a boiling water bath, cooled by immersion in ice for 5 sec, and the rate of renaturation measured at 60° by monitoring the 260 nm absorption in a Gilford spectrophotometer. The absorption at infinite time \( (A_\infty) \) was taken to be the absorption at 60° before denaturation. The renaturation rate constant \( k_2 \) is obtained from the plot of \( 1/(A-A_\infty) \) with time using the relationship of Wetmur and Davidson (1968) whereby,
The kinetic complexity in daltons (N_D) is calculated from the equation of Wetmur and Davidson (1968) for k_2 values in 1M NaCl

\[
\frac{1}{(A-A^\infty)} = 2.04 \times 10^4 k_2 t(\text{sec}) + \frac{1}{0.36 A^\infty}
\]

\[
N_D = 5.5 \times 10^8 \frac{S_{20w}^{PH13}}{k_2} 1.25
\]

(xvii) **Sedimentation analysis**

(Performed by Dr. J. Peacock, CSIRO, Canberra). The sedimentation rate of the sheared DNA used in the analysis was determined in a Beckman analytical ultracentrifuge in 0.05M NaOH, 0.95 M NaCl using a single sector synthetic boundary cell centrifuged at 44,000 r.p.m. Sedimentation coefficients were calculated from the centrifugation data according to Studier (1965).
APPENDIX II

A REAPPRAISAL OF PETITE-POSITIVE AND NEGATIVE YEASTS
Introduction

Recently, a new taxonomic study of yeasts has been published (Lodder 1970) which has radically altered and simplified the previous classification. Using this new classification system, the previous works on typing yeasts as either petite-positive or petite-negative (Bulder 1964A, De Deken 1961, 1966) have been reappraised and the results are presented in Tables I and II. This study has formed the basis for selecting both petite-positive and petite-negative yeasts for further investigation.
### TABLE I

*Petite-positive yeasts*

<table>
<thead>
<tr>
<th>Bulder's yeasts</th>
<th>De Dekken's yeasts</th>
<th>Lodder's Classification</th>
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<tr>
<td><strong>A. Ascomycetous:</strong></td>
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<tr>
<td>Saccharomyces cerevisiae</td>
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<td>S. cerevisiae</td>
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<td>S. turbidans</td>
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<td>S. chevalieri</td>
<td>S. chevalieri</td>
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<td>P. fermentans</td>
<td>(Petite-ve (Bulder))</td>
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<td>Schiz. pombe</td>
<td>(Petite-ve (Bulder))</td>
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<td>Bulder's yeasts</td>
<td>De Deken's yeasts</td>
<td>Lodder's Classification</td>
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<td>B. Asporogenous:</td>
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<td><em>Torulopsis holmii</em></td>
<td>T. holmii</td>
<td>T. holmii (imperfect S. exigus)</td>
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TABLE II
Petite-negative yeasts

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TABLE II (Cont.)

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<td>C. macedoniensis</td>
<td>C. macedoniensis (Imperfect k. marxianus)</td>
<td></td>
</tr>
<tr>
<td>C. parapsilosis</td>
<td>C. parapsilosis (Imperfect Lod. elongisporous)</td>
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</tbody>
</table>
### TABLE II (Cont.)

<table>
<thead>
<tr>
<th>Bulder's yeasts</th>
<th>De Deken's yeasts</th>
<th>Lodder's Classification</th>
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<tr>
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<tr>
<td><strong>B. Asporogenous</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. pelliculosa</td>
<td>C. pelliculosa</td>
<td>(Imperfect H. anomala)</td>
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<tr>
<td></td>
<td>C. pseudotropicalis</td>
<td>(Imperfect K. fragilis)</td>
</tr>
<tr>
<td>C. pulcherrima</td>
<td>C. pulcherrima</td>
<td>(c.f. Metschnikowia pulcherrima)</td>
</tr>
<tr>
<td>C. reukaufii</td>
<td>C. reukaufii</td>
<td>(c.f. Metschnikowia reukaufii)</td>
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<tr>
<td>C. solani</td>
<td>C. solani</td>
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<td>C. stellatoidea</td>
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<td>C. tenuis</td>
<td>C. tenuis</td>
<td></td>
</tr>
<tr>
<td>C. tropicalis</td>
<td>C. tropicalis</td>
<td></td>
</tr>
<tr>
<td>C. utilis</td>
<td>C. utilis</td>
<td>(Imperfect H. jadinii (?))</td>
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<tr>
<td><em>Kloeckera apiculata</em></td>
<td>Kl. apiculata</td>
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</tr>
<tr>
<td><em>Kl. corticis</em></td>
<td>Kl. corticis</td>
<td>(Imperfect H'spora osmophila)</td>
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<td><em>Kl. magna</em></td>
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<td>Kl. javanica</td>
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<td><em>Kl. antillarum</em></td>
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<td><em>Kl. jensenii</em></td>
<td></td>
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<td><em>Kl. lafarii</em></td>
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<td></td>
</tr>
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<td><em>Torulopsis colliculosa</em></td>
<td>T. colliculosa</td>
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<td>Bulder's yeasts</td>
<td>De Deken's yeasts</td>
<td>Lodder's Classification</td>
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<td>T. dattila</td>
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<td>T. dattila</td>
</tr>
<tr>
<td>T. etchellsii</td>
<td></td>
<td>T. etchellsii</td>
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<tr>
<td>T. globosa</td>
<td></td>
<td>T. globosa (Imperfect Citeromyces matritensis)</td>
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<tr>
<td>T. magnoliae</td>
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<td>T. magnoliae</td>
</tr>
<tr>
<td>T. molischiana</td>
<td></td>
<td>T. molischiana (Imperfect H. capsulata)</td>
</tr>
<tr>
<td>T. sake</td>
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<td>C. sake</td>
</tr>
<tr>
<td>T. sphaerica</td>
<td></td>
<td>T. sphaerica (Imperfect K. lactis)</td>
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<tr>
<td>T. stellata</td>
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<td>T. stellata</td>
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<td>T. bacillaris</td>
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<tr>
<td>T. stellata var. cambresieri</td>
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<td>S. rosei</td>
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<td>T. versatilis</td>
<td></td>
<td>T. versatilis</td>
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<tr>
<td>T. anomala</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Trichosporon fermentans</td>
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<td></td>
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<td>Tr. fermentans</td>
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</tbody>
</table>
TABLE III

Response of species of the genus Saccharomyces to petite inducing agents

The data is taken from Bulder (1964A), De Deken (1961, 1966) and van der Walt (1970A). The species are listed according to the new classification (Table I) and listed in groups as per van der Walt (1970A).

Group I - "Saccharomyces sensu stricto"

The first group comprises species closely related to S. cerevisiae. On the basis of fermentative and assimilatory properties, there appear to be three subsidiary lines of development in the 17 species included in this first group. Of the species shown in their sub-groups, only six have been tested and all these are petite-positive (+) denotes petite-positive). The species are:

<table>
<thead>
<tr>
<th>a</th>
<th>b</th>
<th>c</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. norbensis</td>
<td>S. globosus</td>
<td>S. aceti</td>
</tr>
<tr>
<td>S. hienipiens</td>
<td>S. chevalieri (+)</td>
<td>S. capensis</td>
</tr>
<tr>
<td></td>
<td>S. coreanus</td>
<td>S. prostoerdovii</td>
</tr>
<tr>
<td></td>
<td>S. oleaceus</td>
<td>S. heterogenicus (+)</td>
</tr>
<tr>
<td></td>
<td>S. oleaginosis</td>
<td>S. bayanus (+)</td>
</tr>
<tr>
<td></td>
<td>S. italicus (+)</td>
<td>S. inusitatus</td>
</tr>
<tr>
<td></td>
<td>S. cerevisiae (+)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>S. diastaticus</td>
<td></td>
</tr>
<tr>
<td></td>
<td>S. uvarum (+)</td>
<td></td>
</tr>
</tbody>
</table>
TABLE III (Cont.)

Group II:

The second group comprises species closely related to *S. bailii*. All four species in this group have been found to be petite-negative ((-) denotes petite-negative). These species are:

- *S. bailii* (-)
- *S. bisporus* (-)
- *S. rouxii* (-)
- *S. kluyveri* (-)

Group III:

The third group consists of species closely related to *S. rosei*. On the basis of fermentative and assimilatory properties, the nine species comprising the group appear to have two subsidiary lines of development. Also assigned to this group for practical reasons, is the strongly fermentative species that was *Debaryomyces globosus* and is now termed *S. kloeckerianus*. Of the original nine species in the group, five have been subjected to petite-inducing agents and all five are petite-negative, whilst the additional species *S. kloeckerianus* is petite-positive. The species in their sub-groups are:

<table>
<thead>
<tr>
<th>a</th>
<th>b</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. delbrueckii</em> (-)</td>
<td><em>S. inconspicuus</em></td>
</tr>
<tr>
<td><em>S. vafer</em></td>
<td><em>S. rosei</em> (-)</td>
</tr>
<tr>
<td><em>S. microellipsodes</em></td>
<td><em>S. fermentati</em> (-)</td>
</tr>
<tr>
<td>(-)</td>
<td></td>
</tr>
<tr>
<td><em>S. pretoriensis</em></td>
<td><em>S. eupagyeus</em></td>
</tr>
<tr>
<td><em>S. florentinus</em> (-)</td>
<td><em>S. kloeckerianus</em> (+)</td>
</tr>
</tbody>
</table>
TABLE III (Cont.)

Group IV:

The fourth group is phylogenetically heterogeneous and comprises species for which there is little or no evidence of any connection or relationship. Of the ten species listed, four have been found to be petite-positive and a fifth petite-negative. These species are:

- S. unisporus (+)
- S. amurcae
- S. cidri
- S. exigus (+)
- S. montanus (-)
- S. transvaalensis (+)
- S. telluris (+)
- S. dairensis
- S. mrakii
- S. saitoanus

Discussion

From Table I, it can be seen that in the ascomycetous yeasts, there are only five species of Saccharomyces that both workers report to be petite-positive, these are:

- S. cerevisiae
- S. uvarum
- S. bayanus
- S. chevalieri
- S. italicus

In addition to these, Bulder (1964) reports two other Saccharomyces species as petite-positive:
and De Deken (1961, 1966) reports a further *Saccharomyces* species as petite-positive:

*S. kloeckerianus.*

Additionally De Deken reports that the ascomycetous yeast *Nematospora coryli* is also petite-positive.

In the asporogenous yeasts, only one yeast was tested by both workers and found to be petite-positive, this species is *Torulopsis glabrata*. The other yeasts classified as petite-positive by De Deken, were shown to be petite-negative by Bulder. However, there are four other asporogenous yeasts classified as petite-positive by Bulder. These four are:

*Torulopsis holmii*
*Kloeckera africana*
*Brettanomyces clausenii*
*Brettanomyces anomalus*

The first of these yeasts, *T. holmii* has been shown to be the imperfect form of *S. exigus* which is itself petite-positive.

Of the ascomycetous yeast strains found to be petite-positive all, apart from *N. coryli* (De Deken 1961), belong to the genus *Saccharomyces*. However, the genus *Saccharomyces* is phylogenetically heterogeneous and four groups are recognised in the 41 species comprising *Saccharomyces sensu lato* (van der Walt 1970A). The petite-positive *Saccharomyces* species are found to be contained in Group I, *Saccharomyces sensu stricto*, (Table III) and all closely resemble the type
In choosing the petite-negative yeasts from which to study mt DNA, those strains most frequently used in other genetic and biochemical studies and known to be petite-negative (Table II) were chosen. These strains were \textit{K. lactis}, \textit{H. wingei}, \textit{Schiz. pombe} and \textit{C. parapsilosis}. For petite-negative species of the genus \textit{Saccharomyces}, representatives of Groups II and III (Table III) were chosen, these being \textit{S. rouxii} and \textit{S. rosei} respectively. The results of these studies on petite-negative yeasts are presented in Chapter 2 of this thesis.

In choosing petite-positive species only one ascomycetous yeast, \textit{N. coryli}, did not closely resemble \textit{S. cerevisiae}. However, \textit{N. coryli} is a plant pathogen, having been isolated from diseased cotton bolls and diseased hazlenuts, and is not found in Australia. Hence permission to import the strain was refused by the General, Animal and Plant Quarantine Divisions, Commonwealth of Australia (File No. 74/1377, B63182, 6 August 1974). Thus only asporogenous, petite-positive yeast strains were available. As \textit{T. holmii} is the imperfect form of \textit{S. exiguis}, which it was felt resembled \textit{S. cerevisiae} in being of the same genus and petite-positive, this yeast was left out of the study. Hence there remained four asporogenous, petite-positive yeasts, (\textit{T. glabrata}, \textit{Kl. africana}, \textit{Br. anomalous} and \textit{Br. clausenii}) which were obtained and analysed, the results being presented in Chapter 3 of this thesis.
REFERENCES


J. Bact. 119, 1063-1068.

Howell, N., Molloy, P.L., Linnane, A.W. and Lukins, H.B.

Huang, M., Biggs, D.R., Clark-Walker, G.D. and Linnane, A.W.


Mut. Res. 29, 67-76.

Acta 222, 611-620.


695-719.


770-779.


U.S.A. 69, 1830-1834.


Eur. J. Biochem. 53, 137-144.


ADDENDA

Page

1 (line 7) Mykes
2 (line 12) Stier
4 (line 29) cytochrome c₁
5 (line 6) Saltzgaber
10 (line 16) Leishmania; (line 24) Tewari
19 (line 19) mikamycin
24 (line 4) ... to EtBr the more extensive is the decrease
35 (line 6) maintained
Fig. 1-6 wild-type refers to the pet⁺[rho]⁺ parent
strain from which the respiratory-deficient
mutants arose
57 (line 5) respectively
65 (line 21) EDTA
68 (line 2) buoyant density
72 (line 17) Tewari
73 (line 2) 1.689 g/cm³
76 (line 18) Schimmel cultures
89 (line 7) and
120 (line 14) viability genes
127 (line 25) the log₁₀ of the spontaneous
Fig. 4-5 left ordinate add: log₁₀ petite also change -1. to -1.
136 (line 20) anomalous
167 (line 30) add: Chattoraj, D.K., Younghusband, H.B. and
139-149.
180 (line 20) add: Wheelis, L., Trembath, M.K. and Criddle, R.S.
838-845.