STUDIES ON INTERSPECIFIC CELL FUSION IN YEAST

by

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Statement

I declare that the results presented in this thesis are my own and were obtained under the supervision of Dr. G. D. Clark-Walker. The material contained in this thesis has not been presented for award of any other degree or diploma in any University. Part of this work has been published:


Cesira L. Galeotti
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Synopsis

The recent acquisition of techniques which allow fusion between cells of distantly related organisms has provided new possibilities for studying interactions between different genomes. Application of these techniques to the examination of petite negativity in yeast has been investigated in this study. In recent years, the knowledge acquired on structure and genetic content of yeast mtDNA has indicated that the reason why petite-negative yeasts cannot sustain the petite mutation is likely to reside with differences between the nuclear genomes of petite-positive and petite-negative species, rather than between their mitochondrial genomes. This point could be resolved by fusing a petite-negative species with a \( \rho^0 \) mutant of a petite-positive one. The approach taken in this work was to analyse first fusion between two supposedly related petite-positive yeasts and subsequently between a petite-negative and a petite-positive species. It was found that fusion products could not be isolated between *S. cerevisiae* and another petite-positive yeast, *S. unisporus*, although intraspecific fusion was achieved in both species. On the contrary, fusion products were obtained when a double auxotroph from the petite-negative *K. lactis* was fused with two different \( \rho^0 \) mutants of *S. cerevisiae*. Discordant results as well as common features were observed in the two classes of fusion products. While fusion between the *K. lactis* strain and a *S. cerevisiae* mutant carrying an arg8 mutation gave rise to colonies with the *K. lactis* phenotype and, in
addition, a requirement for arginine, fusion between the same *K. lactis* auxotroph and another *S. cerevisiae* mutant produced prototrophic colonies. In both cases only portions of the *S. cerevisiae* genome were retained in a predominantly *K. lactis* background. Evidence is given that a transposition is possibly responsible for the *arg* phenotype of the first class of fusion products. A more complex condition is present in the prototrophs isolated from the other fusion experiment. "Segregants" from one of these fusion products, KF4, show acquisition of expression of some *S. cerevisiae* characters which were unexpressed in KF4. One of these characters is the ability to produce ρ' mutants.

From the results presented in this thesis two important deductions can be made. Firstly, cell fusion techniques can provide the means for analysing interrelations between genomes of different species as well as between different genomes within the same organism. Secondly, complete loss of mtDNA in a petite-negative yeast can be induced without concurrent loss of cell viability in fusion products containing nuclear information from a petite-positive species.
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<tr>
<td>bp</td>
<td>base pair</td>
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<tr>
<td>CsCl</td>
<td>caesium chloride</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>mtDNA</td>
<td>mitochondrial deoxyribonucleic acid</td>
</tr>
<tr>
<td>rDNA</td>
<td>ribosomal deoxyribonucleic acid</td>
</tr>
<tr>
<td>E.B.</td>
<td>ethidium bromide</td>
</tr>
<tr>
<td>fg</td>
<td>femtogram (10^{-15} gram)</td>
</tr>
<tr>
<td>μl</td>
<td>microlitre (10^{-6} litre)</td>
</tr>
<tr>
<td>μm</td>
<td>micrometre (10^{-6} metre)</td>
</tr>
<tr>
<td>nm</td>
<td>nanometre (10^{-9} metre)</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethilene glycol</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>rDRNA</td>
<td>ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>SSC</td>
<td>standard saline citrate</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
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**RESULTS**

A. *Isolation of fusion products between D6ep~ and K. lactis*  
B. *Characterisation of fusion products between D6ep~ and K25*

1. Analysis of physiological characters  
2. Analysis of DNA content and mitotic segregation  
3. Analysis of specific DNA sequences in fusion products by molecular probing  
4. Genetic analysis of fusion products

**DISCUSSION**
CHAPTER 3.  FUSION BETWEEN S. CEREVISIAE Fe6− AND K. LACTIS

INTRODUCTION

MATERIALS AND METHODS

Preparation and fusion of protoplasts

Analysis of physiological characters

X-ray irradiation and benlate

Isolation of purified mtDNA and nuclear DNA

Ethidium bromide production of petite colonies

RESULTS

A. Isolation of prototrophic colonies between Fe6− and K. lactis

B. Analysis of prototrophic colonies

(1) Physiological characteristics

(2) Analysis of X-ray sensitivity

(3) Characterisation of KF4.1r and KF4.2r

(4) Analysis of specific DNA sequences in prototrophs by molecular cloning

C. Induction of the petite mutation in prototrophic clones and their segregants

D. Spontaneous petite formation in KF4.1r and KF4.2r

DISCUSSION

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GENERAL INTRODUCTION
A. THE MITOCHONDRIAL GENOME OF YEAST

1. *Molecular structure of mt DNA in S. cerevisiae*

Self-replicating covalently closed circular molecules of DNA are present within the mitochondria of *S. cerevisiae* at the average number of 50 per haploid cell (Williamson *et al.*, 1978). The size of a mtDNA molecule in this organism varies between different strains ranging from 67,000 to 78,000 bp (Borst *et al.*, 1977; Prunell *et al.*, 1977; Morimoto *et al.*, 1978). As for the primary structure of mtDNA, it was found that sequences are heterogenously organised since long AT-rich stretches, with a GC content lower than 5%, are interspersed with GC-rich regions (Bernardi *et al.*, 1968, 1972). The AT-rich sequences, called spacers, account for about 50% of the DNA (MW 4 x 10^5 d) and possibly do not contain any genetic information. It has been proposed that spacers could provide regions of partial homology for frequent recombinational events which would account for the rapid evolution of the mitochondrial genome in yeast (Clark-Walker and Miklos, 1974; Prunell and Bernardi, 1974). GC-rich regions comprise sequences with 23-34% GC content which are known to code for specific mitochondrial functions (Macino and Tzagoloff, 1979a, 1980; Hensgens *et al.*, 1979; Coruzzi and Tzagoloff, 1979). Also interspersed in AT-rich regions
there exist short GC clusters which contain up to 79% GC. Inverted repeated sequences have been found in GC clusters and it has been speculated that these may be involved in the processing of RNA transcripts (Macino and Tzagoloff, 1979a; Nobrega and Tzagoloff, 1980; Tzagoloff et al., 1980).

Great interest on the structure of mtDNA stemmed firstly from the discovery that a mutation, called petite, peculiar to some yeast species (petite-positive species) is due to deletions or total loss of mtDNA.

2. The petite mutation

Gross alterations in the composition of mt DNA in yeast produce a characteristic phenotype originally described for its macroscopic appearance as petite colonie (Ephrussi, et al., 1949a). Loss of mitochondrial function results in the formation of small colonies on medium containing a limiting amount of a fermentable carbon source. The petite phenotype was ascribed to mutation at an extrachromosomal genetic determinant, called ρ (Sherman, 1964) and later identified as mt DNA (Mounolou et al., 1966; Goldring et al., 1970; Nagley and Linnane, 1970), when it was found that the mutation was inherited in a non-Mendelian fashion, it occurred spontaneously at very high frequency (1% per generation in S. cerevisiae), haploid and diploid cells showed the
same mutation rate and it never reverted (Ephussi et al., 1949b; Ephrussi and Hottinguer, 1950).

Biochemical studies on the physiological defects produced by the mutation established that these resided with the mitochondrial respiratory-chain system. Petites lack mitochondrial protein synthesis and this results in a pleiotropic absence of several components of respiratory enzyme complexes and loss of mitochondrial oxidative phosphorylation (Schatz and Saltzgaber, 1969; Kuzela and Grecna, 1969).

Although this phenotype is the same in petites of different origin, the changes in mt DNA structure that accompany the petite phenotype are not all of one kind. Petites can either have lost all of the mt DNA ($\rho^0$) or retained a continuous segment of the wild-type mt DNA ($\rho^-$). Portions varying between 0.1 and 80% of the wild-type mitochondrial genome are conserved in $\rho^-$ mutants and reiterated to form defective molecules. The stretch of DNA retained is amplified, that is tandem or palindromic repeats are joined to form circular molecules of heterogeneous size, or oligomers of the conserved sequence (Locker et al., 1974; Lazowska and Slonimiski, 1976, 1977). More recently restriction enzyme analysis and electron microscopy studies on the sequence organisation in ten petites have shown that some $\rho^-$ mt DNAs have mixed tandem and palindromic repeats. Also only a few molecules, in all $\rho^-$ mt DNAs examined, were circular (Heyting et al., 1979b).
Complete loss of mt DNA has first been reported in petite mutants obtained by treatment of wild type cells with acriflavine (Moustacchi and Williamson, 1966). In fact the two acridine euflavin and proflavine, of which acriflavine is a mixture, were later shown to induce petites mainly of the $\rho^0$ type (Mattik and Nagley, 1977).

Other drugs and mutagens were also found to induce petites at very high frequency. The intercalating dye ethidium bromide and its analogues are very effective in producing lesions specifically of the mt DNA (Slonimski et al., 1968; Borst, 1972; Mahler, 1973; Hall et al., 1977). Synthesis and transcription of mt DNA are blocked by ethidium bromide and degradation of the DNA follows rapidly in metabolically active cells (Fukuhara and Kujawa, 1970; Goldring et al., 1970; Perlman and Mahler, 1971; Criddle et al., 1976).

Ultraviolet radiation is another efficient petite inducer (Raut and Simpson, 1955; Maroudas and Wilkie, 1968; Moustacchi, 1969, 1972). Pyrimidine dimer formation is one of the events known to be responsible for initiating the reactions leading to large deletions of mt DNA (Moustacchi and Enteric, 1970; Prakash, 1975; Heude and Moustacchi, 1979). Unlike ethidium bromide, U.V. radiation is not a specific mutagen for mt DNA and it probably acts by causing defects in nuclear functions necessary for mt DNA maintenance (Johnson et al., 1973).
Acridines, ethidium bromide and U.V. radiation are the most widely used mutagens for petite induction but a large number of other physical or chemical treatments can lead to the petite mutation. However, the most striking feature on rate of mutability of mt DNA in *S. cerevisiae* rests with the high spontaneous frequency of petite formation. The most recent and comprehensive hypothesis advanced on the petite mutation, which considers the high spontaneous rate of petites as well as other peculiarities of mitochondrial genetics, has been formulated by Clark-Walker and Miklos (1974). These workers proposed that the molecular organisation of the mt DNA itself, possessing genes spaced by AT-rich sequences, could originate excision and insertion events at high frequency if regions of partial homology are provided by the AT-rich segments interspersed along the circular DNA molecule.

A less elaborate but similar hypothesis was advanced also by Bernardi (Prunell and Bernardi, 1974).

3. Genetic structure of mt DNA in *S. cerevisiae*

Point mutations or small deletions that do not grossly alter the structure of mt DNA have been characterised and extensively used for mapping the mitochondrial genome of *S. cerevisiae*. Three classes of such mutations have been reported.
Antibiotic-resistant mutants - The identification of genetic loci which confer resistance to antibiotics that inhibit mitochondrial protein synthesis or respiration has provided the first means for mapping mitochondrial genes. Resistance to erythromycin (\(ery^R\)) and chloramphenicol (\(cap^R\)) were the first mitochondrial markers to be identified (Thomas and Wilkie, 1968; Linnane et al., 1968). Comparative studies on loss or retention of \(ery^R\) and \(cap^R\) loci in \(\rho^{-}\) mt DNAs and hybridisation pattern of these DNAs with mitochondrial rRNA later established that the sequence coding for 21S rRNA lies in the region where the \(ery^R\) and \(cap^R\) markers had been mapped (Fukuhara et al., 1974; Faye et al., 1974; Nagley et al., 1974). Similarly mutants resistant to paromomycin (\(par^R\)) were used to genetically define the position of the 15S rRNA gene (Faye et al., 1975).

Two mutations conferring resistance to oligomycin (\(olil\) and \(olil2\)) and located at distant loci also proved valuable in identifying mitochondrial genes. Subunit 9 of the ATPase complex was shown to be coded for by the \(olil\) structural gene (Groot Obbink et al., 1976; Wachter et al., 1977; Macino and Tzagoloff, 1979) while the \(olil2\) region specifies subunit 6 of the same complex (Griffiths and Houghton, 1974; Roberts et al., 1979; Macino and Tzagoloff, 1980).
Finally several mutants resistant to antimycin, diuron, mucidin and funiculosin, all specific inhibitors of the coenzyme QH$_2$-cytochrome c reductase activity, have been characterised and mapped in the region of mt DNA where the cytochrome b deficient mutants are located (Subik, 1975; Lang et al., 1976; Colson et al., 1977; Colson and Slonimski, 1979; Subik and Goffeau, 1980).

*Mit* and *Syn* mutants - Mutations that alter the expression of a particular mitochondrial gene are called *mit* or *syn* depending on whether components of the respiratory chain system (*mit*) or of the mitochondrial protein synthesis system (*syn*) are affected. Since *syn* mutations halt mitochondrial protein synthesis, their phenotype does not differ from the *petite* phenotype except for the case of conditional *syn* mutants, which can be distinguished from *petites* at the permissive conditions (Tzagoloff et al., 1975b; Slonimski and Tzagoloff, 1976).

Complementation studies of several *mit* mutants together with biochemical analysis of functions lost in a particular *mit* mutant resulted in precise localisation and resolution of the complex structure of the genes coding for cytochrome b and for the three subunits of cytochrome oxidase (Slonimski and Tzagoloff, 1976; Foury and Tzagoloff, 1978; Slonimski *et al.*, 1978).
Syn\textsuperscript{−} mutants have been employed for mapping genes coding for mitochondrial tRNAs. Although the approximate gene location for some tRNA species had been determined by tRNA-DNA hybridisation studies and deletion mapping (Casey \textit{et al.}, 1974; Fukuhara \textit{et al.}, 1976; Martin, N.C. \textit{et al.}, 1977; Wesolowski and Fukuhara, 1979), only the isolation of \textit{syn}\textsuperscript{−} mutants with lesions at specific tRNA loci could allow positioning of these genes by genetic techniques (Trembath \textit{et al.}, 1977; Martin, R. \textit{et al.}, 1977; van Ommen \textit{et al.}, 1977; Berlani \textit{et al.}, 1980).

Genetic studies involving the use of these different classes of mutants combined with physical methods, such as RNA-DNA or DNA-DNA hybridisation and restriction enzyme analysis, have contributed to the establishment of a genetic map of mt DNA. It is now apparent that only a limited number of mitochondrial components is coded for by the mitochondrial genome.

Ribosomal RNA – Both large and small subunits of the mitochondrial rRNA are specified by a mitochondrial gene. The 15S rRNA locus is separated from the 21S rRNA locus by at least 25,000 bp (Sanders \textit{et al.}, 1975; Faye \textit{et al.}, 1975; Sriprakash \textit{et al.}, 1976). Moreover the gene for the 21S rRNA sometimes shows a complex organisation. An insert of about 1,000 bp splits this locus (Bos \textit{et al.}, 1978). This intervening sequence coincides with a polarity locus called \(\omega\) (Bolotin \textit{et al.},
1971; Netter et al., 1974) that affects the transmission frequency of markers in this region. Deletion of the insert is accompanied by loss of polarity of recombination in the 21S rRNA region (Dujon et al., 1976; Borst et al., 1977; Jacq et al., 1977; Sanders et al., 1977; Faye et al., 1979; Dujon, 1980).

A detailed physical map of the 21S and 15S rRNA loci has been recently constructed by restriction enzyme analysis (Morimoto et al., 1978; Heyting et al., 1979a, b; Heyting and Menke, 1979).

tRNAs - The majority of the 24 tRNA genes identified so far are sparsely located between the two rRNA loci (Fukuhara et al., 1976; Trembath et al., 1977). Only 3 tRNA species have been found in other regions of the map (Wesolowski and Fukuhara, 1979; Tzagoloff et al., 1979, 1980).

The nucleotide sequence of most tRNA genes has been determined (Martin, R. et al., 1978; Bos et al., 1979; Li and Tzagoloff, 1979; Miller et al., 1979; Berlani et al., 1980). The data obtained from the sequence analysis of these genes confirmed what was already evident from comparing protein and DNA sequences for some mitochondrial gene products, that is the genetic code in yeast mtDNA differs from the universal code in that UGA is recognised as a tryptophan codon and CUA as a threonine rather than a leucine codon (Coruzzi and
ATPase complex - The structural genes for two subunits of the oligomycin-sensitive ATPase complex have been unambiguously mapped. \textit{olil} is located between the 21S rRNA and the cytochrome b locus and it specifies subunit 9 of the complex (Wächter et al., 1977; Coruzzi et al., 1978). More recently this was confirmed by DNA sequencing data on a portion of the \textit{olil} gene (Macino and Tzagoloff, 1979). The DNA sequence of a \textit{petite} retaining the \textit{olil} marker was in good agreement with the known primary structure of the protein (Wächter et al., 1977).

The gene for subunit 6 has been assigned to the \textit{oli2} locus, which lies close to the cytochrome oxidase subunit I gene (Choo et al., 1977; Roberts et al., 1979).

Apocytochrome b - A large segment (8,000 bp) of the region spanning between the \textit{olil} and the \textit{oli2} loci is occupied by the split gene coding for cytochrome b. The gene is composed of six exons separated by five introns (Slonimski et al., 1978; Dujon, 1979). Two long open reading frames are present in introns. They were called \textit{box3} and \textit{box7} because of their localisation within the two regions genetically identified by the \textit{box3} and \textit{box7} \textit{mit}^− mutations (Kreike et al., 1979; Solioz and Schatz, 1979; Nobrega and Tzagoloff, 1980). \textit{Box3} starts at the distal end of intron 1 (\textit{I}_1) and adjoins intron 2(\textit{I}_2),
while box7 is all within intron 4(I4) and continuous with exon 4 (E4) (Lazowska et al., 1980; Alexander et al., 1980; van Ommen et al., 1980). The finding that transcripts larger (23S-34S) than the 18S RNA presumed to be the mRNA for cytochrome b are observed in box3 and box7 mutants and also new polypeptides, sometimes larger than cytochrome b, that are accumulated in such mutants give strong evidence to the hypothesis that the products of the open reading frames in I2 and I4 are "maturases" involved in RNA splicing (Kreike et al., 1979; Solioz and Schatz, 1979; Haid et al., 1980; Lazowska et al., 1980). An interesting peculiarity of some of these intron mutations is that they also affect synthesis of sub. I of cytochrome oxidase (Kotilak and Slonimski, 1976; Claisse et al., 1978, 1980; Church et al., 1979).

However, as for the intervening sequence in the large rRNA gene, not all S. cerevisiae strains have the first 3 introns (Sanders et al., 1977; Grivell et al., 1979; Nobrega and Tzagoloff, 1980). Expression of the 'short' cytochrome b gene is not affected by the absence of I1-3, that are therefore called 'optional' introns (Borst, 1981).

Cytochrome c oxidase - Three genetically unlinked regions of the mt DNA contain the genes coding for the three larger subunits of this complex. The oxi1 and oxi2 sequences, which code for subunit II and subunit III
respectively, are located between the \( cap^E \) (21S rRNA) and \( par^E \) (16S rRNA) loci (Slonimski and Tzagoloff, 1976; Cabral et al., 1978; Fox, 1979; Thalenfeld and Tzagoloff, 1980; Stephenson et al., 1981) and are separated by at least two tRNA genes (Trembath et al., 1977; Wesolowski and Fukuhara, 1979). The gene coding for subunit I, \( oxi3 \), lies between the \( par^E \) and \( oli2 \) markers (Slonimski and Tzagoloff, 1976; Grivell and Moorman, 1977; Eccleshall et al., 1978). \( oxi3 \) is the most studied of the three genes since, like the gene for cytochrome b, it is split by intervening sequences (Borst and Grivell, 1978). Moreover, an unusual mechanism of regulation is thought to be responsible for the lack of expression of \( oxi3 \) in intron mutants of cytochrome b (Claisse et al., 1980).

To date the structural gene for subunit I is known to be interrupted by 7 or 8 introns, four of which contain long open reading frames (Tzagoloff et al., 1980).

\( \text{var1} \) - A protein termed \( \text{var1} \) because of its variable electrophoretic mobility among different \( \rho^+ \) strains is also encoded by the mitochondrial genome (Douglas and Butow, 1976). Genetic analysis of the polymorphic forms of \( \text{var1} \) (MW 40,000-44,000) aided by restriction mapping has permitted the positioning of the \( \text{var1} \) locus to a 2,100 bp DNA fragment located between the \( \text{ery}^E \) and \( \text{oli1} \)
markers (Butow et al., 1977; Perlman et al., 1977). It is now established that var1 represents the structural gene for a mitochondrialy translated component of the 37S ribosome subunit (Terpstra et al., 1979; Groot et al., 1979). However sequencing of the var1 locus has failed to show the gene (Tzagoloff et al., 1980).

4. Nuclear genes which affect mitochondria

It is clear from the small number of mitochondrial encoded gene products contributing to mitochondrial biogenesis that many nuclear genes must be involved in the maintenance of this organelle. Early studies described about 40 unlinked nuclear loci (pet) that, when mutated, produce a respiratory-deficient phenotype which can be distinguished from the cytoplasmically determined petite phenotype by its Mendelian pattern of inheritance (Sherman, 1963; Sherman and Slonimski, 1964; Kotylak, 1973). Among these a chromosomal mutation op1, should be mentioned. op1 renders $\rho^-$ mutations lethal to the cell (Kováčova et al., 1968). Thus, all mitochondrial respiratory-deficient mutants isolated in op1 strains are of the mit$^-$ type. This proved most useful for selecting large numbers of mit$^-$ mutants subsequently used for mapping the box and oxi regions of the mt DNA (Kotylak and Slominski, 1976; Schweyen et al., 1978). In an analogous way nuclear conditional mutants (tsp) which produce $\rho^-$ petites at high frequency at non-permissive
temperature were employed for ordering mitochondrial markers by studying their transmission in several $\rho^-$ clones (Backhaus et al., 1978; Schweyen et al., 1978). The temperature-sensitive mutants used in these studies were only a few among the 270 $te$ $pet$ mutants isolated by Burkl et al. (1976) and attributed to 106 different complementation groups.

At least five nuclear mutations have been reported to specifically block the synthesis of the mitochondrial cytochrome oxidase subunits (Ebner et al., 1973) and several were found which affect synthesis of cytochrome b or subunits of the oligomycin-sensitive ATPase (Tzagoloff et al., 1975a).

Attempts have been made also to characterise nuclear genes involved in mt DNA metabolism. To this end the effects on mt DNA of mutations known to affect nuclear DNA metabolism have been investigated. Some of these nuclear mutations were shown to increase the spontaneous mutation frequency of mt DNA (Moustacchi et al., 1976). In particular two mutations, $cdc8$ and $cdc21$, which lead to defective DNA replication also produce petites at a higher rate (Newlon and Fangman, 1975). However, most petites isolated from $cdc8$ or $cdc21$ mutants still contain mt DNA indicating that the replication of mt DNA is not affected (Newlon et al., 1979). Another conditional mutation of nuclear origin called $tpi$ has been described to cause a reduction of mt DNA synthesis. At the non-
permissive temperature tpi cells are converted at high frequency into petites which lack mt DNA (Rubin and Blamire, 1979).

Other aspects of mt DNA metabolism, such as repair and recombination pathways, are also being investigated in an attempt to gain a better understanding of the mechanisms which produce defective mt DNA molecules in petites. Since enzymes involved in the repair of mt DNA after mutagenic treatment could also be responsible for the high spontaneous mutability of mt DNA, mutations that alter the rate of petite induction by U.V. light or ethidium bromide have been studied. Impairment of a nuclearly encoded function involved in replication repair specifically at the mt DNA level has been proposed to be responsible for the increased U.V. sensitivity towards the induction of petites in the chromosomal mutant uvsρ5 (Moustacchi et al., 1976). Nuclearly inherited mutants called ebi, because they manifest a reduced frequency of petite induction in the presence of ethidium bromide, have been isolated and characterised. Analysis of transmission and recombination frequencies of mitochondrial markers in ebi mutants showed that some of these mutations could introduce a bias of recombination frequencies for some mitochondrial markers (Dujardin and Dujon, 1979).

The identification of nuclear mutators specific for mt DNA has been attempted recently by a different
approach. A large number of X-ray sensitive mutants were tested for increased mutation rate of mitochondrial loci. Five mutator strains were detected (gaml-5) which were of two types. Three were described as general mutators of the mt DNA and did not modify the spontaneous mutation rate of nuclear genes, while the other two affected the mutation frequency of particular mitochondrial loci (Foury and Goffeau, 1979). Mutators similar and possibly allelic to gam strains of the former type have also been reported by Johnston (1979).

Since the complex organisation of the mitochondrial genome has become more apparent, greater interest has been devoted to nuclear genes contributing to the mitochondrial translation system. The acquisition in recent years of several well characterised mit mutants has already allowed the identification of nuclear mutations which act as informational suppressors of mit mutations. One such mutation (sum1) has been found to suppress a nonsense mutation mapped in the cytochrome b locus. sum1 is recessive and its product is probably involved in the mitochondrial translation system (Coruzzi and Tzagoloff, 1980). Screening of several revertants of mit- mutants has resulted in the isolation of two other nuclear suppressors called NAM1 and NAM2. Although NAM1 had been selected to suppress a mit- mutation in oxi3, it showed suppressor activity on mit- mutations of oxi1, oxi2, box7 and box9. Thus, it was suggested that NAM1
could specify a nuclear function involved in mitochondrial translation. In the case of NAM2 only mutations of box7 are suppressed. As the nature of box7 mutations has yet to be resolved, the mechanism of suppression by NAM2 could not be identified (Dujardin et al., 1980).

Unfortunately the many studies that led to the identification of nuclear loci controlling mitochondrial structure and functions have not yet provided a precise description of the functions expressed by such loci. A line of research that could prove most fruitful in this respect has been adopted recently by some workers who introduced the use of transformation techniques for physically isolating and characterising pet genes.

5. Structure of mt DNA in other yeasts

Most of the present knowledge on molecular and genetic structure of mt DNA in yeast has resulted from studies on the mitochondrial genome of S. cerevisiae. However, the examination of properties of mt DNA in other yeasts has revealed that the structure of this DNA is not uniform among different species.

A first division between yeast species in relation to their mitochondrial genome was introduced by Bulder (1964), who discovered that not all yeasts could form petites. He classified as petite-negative all species which did not give rise to petites even after acriflavine
or ethidium bromide treatment. Ethidium bromide has been shown to have the same effects on mt DNA synthesis in petite-negative as in petite-positive yeasts (Luha et al., 1971; Wolf and Del Giudice, 1980) and a transient petite-like phenotype is induced by the drug in petite-negative cells. However these changes are reversed if the drug is removed or else cells lyse under prolonged treatment (Schwab et al., 1971; Crandall, 1973).

The reason why petite-negative yeast cannot sustain petite induction does not lie in energetic considerations since respiratory deficient mutants of nuclear origin can be isolated in petite-negative species (Wolf et al., 1971; Goffeau et al., 1973). In addition point mutations in the mt DNA of the petite-negative Schizosaccharomyces pombe were shown to produce viable respiratory-deficient mutants (Wolf et al., 1976).

Another interesting aspect of yeast mitochondrial genetics was uncovered by the finding that the frequency of spontaneous petite formation varies greatly between different petite-positive species (O'Connor et al., 1976; Clark-Walker et al., 1981a).

Studies on the structure of mt DNA in species other than S. cerevisiae, aimed at providing a better understanding of both phenomena, petite formation and petite-negativity, have been reported. The data attained so far by these studies are briefly summarised.
(1) Structure of mt DNA in petite-positive species - A large variation in size between mt DNAs of different petite-positive yeasts was found when electron microscopy analysis of mt DNA molecules determined that the mitochondrial genome can be as small as 6\(\mu m\) in Torulopsis glabrata (O'Connor et al., 1976) as opposed to 25\(\mu m\) in S. cerevisiae (Hollenberg et al., 1970). Intermediate sizes have been estimated for the mt DNA of Kloekera africana (8\(\mu m\)) and Brettanomyces anomalus (18\(\mu m\)).

Restriction enzyme analysis of the mt DNA from these two species revealed that only a few Hpa-Hae GC-rich sites are present in these yeasts (Clark-Walker and McArthur, 1978), while 60-100 of such sites are found in S. cerevisiae mt DNA (Prunell et al., 1977).

Physical mapping of restriction sites and location of some genes by RNA-DNA and DNA-DNA hybridisation techniques have been undertaken for T. glabrata and K. africana mt DNA. The large and small rRNA genes are separated by only 2,000-2,600 bp in T. glabrata and by about 1,500 bp in K. africana (Clark-Walker et al., 1980, 1981b). Moreover the size of the large rRNA in both yeasts is smaller (2,700 bases) than the large mitochondrial rRNA of S. cerevisiae (Sriprakash and Clark-Walker, 1980).

From these studies it was also discovered that a large inverted duplication (8,600 bp) is present in the
mt DNA of *K. africana*. The duplication contains a partial repeat of the large rRNA sequence (Clark-Walker *et al.*, 1981b).

(2) *Structure of mt DNA in petite-negative species*—Size heterogeneity of mt DNA is again encountered within petite-negative species. The length of mt DNA molecules of five species has been reported to date: *Candida parapsilosis*, 11 μm; *Hansenula wingei* 8 μm; *Kluyveromyces lactis*, 11 μm; *Sch. pombe*, 6 μm (O'Connor *et al.*, 1975) and *Torulopsis calliculosa*, 15 μm (Kojo, 1976). The G + C content of mt DNA, as estimated by buoyant density values, is also diverse in petite-negative species. Moreover, *H. wingei* mt DNA has a buoyant density (1.686 g/cm³) very similar to *S. cerevisiae* (1.684 g/cm³) suggesting that no obvious structural peculiarity can account for petite negativity.

Additional information on mt DNA structure is available only for *K. lactis* and *Sch. pombe*.

Comparative studies have established that there is only approximately 10% sequence homology between the mt DNA of *S. cerevisiae* and *K. lactis* (Groot *et al.* 1975). However the mt DNA from a number of *S. cerevisiae* ρ− mutants, each known to contain only a specific gene sequence, could be used in low stringency DNA–DNA hybridisation experiments for mapping the mt DNA of *K. lactis*. The genes for large and small rRNAs, subunit 9 of the ATPase complex, subunit I of cytochrome
c oxidase and cytochrome b have been ordered on the map. The relative positions of these genes differ from the corresponding gene order in *S. cerevisiae* mtDNA (Groot and Van Harten-Loosbroek, 1980).

As for *S. cerevisiae* the first step towards mapping of mitochondrial genes in *Sch. pombe* was undertaken by recombination analysis between antibiotic-resistance markers. The isolation of extrachromosomal *ery* and *par* mutants in this yeast has been facilitated by the identification of an antimycin resistant mutant (*ana*-8) with 'mutator activity' specific for the mitochondrial genome (Wolf et al., 1976; Del Giudice et al., 1977; Seitz et al., 1977). More recently another mitochondrial mutator strain (*ana*-14) has allowed the isolation of 17 *diu* mutants (Burger and Wolf, 1981).

From this brief summary of the mitochondrial genetics of yeast three points are apparent. Firstly, the recently developed techniques of RNA-DNA, DNA-DNA hybridisation and DNA sequence analysis together with the established genetic and biochemical techniques will soon provide full knowledge of the functions encoded by the mt DNA of *S. cerevisiae* and a few other yeast species. Secondly, major differences are present in the organisation of the mt DNA of different species. Finally, the interactions between the nuclear and the mitochondrial genomes need to be explored more thoroughly.
in order to solve some of the basic problems inherent to \textit{petite} formation in yeast. These are, for example, the different spontaneous \textit{petite} frequencies exhibited by \textit{petite}-positive yeasts and the absence of \textit{petite} formation in \textit{petite}-negative yeasts.

The recent establishment of cell fusion techniques could prove valuable if applied to an examination of nuclear background effects on mt DNA in hybrids. Hence the prime objective of this study was to investigate the possibility of obtaining hybrids between different yeast species by cell fusion.

B. \textbf{CELL FUSION IN MICRO-ORGANISMS}

In bacteria and fungi two non-interbreeding cell types can be induced to fuse provided that their cell-wall is first removed. This is easily attainable by using lytic enzymes (McQuillen, 1960; Spizizen, 1962; Weiss, 1976; Eddy and Williamson, 1957; Villanueva and Garcia Acha, 1971). In the presence of an osmotic stabiliser, cell viability of wall-deprived protoplasts is not greatly affected and, under defined conditions, resynthesis of cell-wall materials can take place in protoplasts thus converting them back to wall-bearing cells (Okanishi \textit{et al.}, 1974; Fodor \textit{et al.}, 1975; Svoboda, 1966; Svoboda and Nečas, 1966; Nečas, 1971; Peberdy, 1976, 1979a).
Once the methods of obtaining protoplasts and their reversion to normal cells had been established, a further step towards the achievement of cell fusion at high frequency was promoted by the discovery that polyethylene glycol (PEG) can induce fusion between the most disparate biological membranes. The fusogenic behaviour of PEG was first reported for plant protoplasts (Kao and Michayluk, 1974; Wallin et al., 1974) and has been later applied to induce fusion in a wide range of biological systems, from bacterial protoplasts to mammalian cells (Ferenczy et al., 1975b; Anné and Peberdy, 1975; Pontecorvo, 1975; Maggio et al., 1976).

1. Cell fusion in prokaryotes

The first successful attempts to fuse bacterial protoplasts with PEG were performed with polyauxotrophic strains of *Bacillus megaterium* (Fodor and Alföldi, 1976) and *Bacillus subtilis* (Schaeffer et al., 1976). In both cases selection for prototrophic colonies was the criterion used for detecting fusion products. Genetic analysis of the primary colonies obtained in *B. megaterium* revealed that these were either colonies whose progeny consisted of mixed populations of different stable recombinant phenotype or uniform colonies composed of only one type of stable recombinant. Some mixed colonies also contained unstable prototrophs (Fodor and Alföldi, 1976). Moreover, the distribution of
recombinant phenotypes was anomalous when direct selection for given recombinant classes was applied. Physiological effects, determined by growth and selection conditions, were found to influence the frequency for some recombinant phenotypes (Fodor and Alföldi, 1979). Similarly, in *B. subtilis* when direct selection for recombinants was employed, the formation of transient diploids was followed by segregation of stable recombinants which could also contain unselected markers. Only a few prototrophs were obtained by selecting fusion products on minimal medium. Since no auxotrophic segregants could be isolated from them, these prototrophs were haploid recombinants (Schaeffer et al., 1976). High-frequency protoplast fusion in *B. subtilis* has also been demonstrated by electron microscopy (Prehel et al., 1979).

An interesting study on *B. subtilis* fusion products has been reported recently by Hotchkiss and Gabor (1980). Several auxotrophic clones obtained by protoplast fusion and exhibiting either of the parental phenotypes were in fact "diploids" containing the unchanged genomes of both partners. The other chromosome was simply not expressed.

Mixed colonies composed of stable prototrophs and stable recombinants have also been observed after protoplast fusion of auxotrophic strains of the gram-negative bacterium *Providencia alcalifaciens* (Coetzee et al., 1979).
Frequent recombination was found as a result of fusion within *Streptomyces* species (Hopwood *et al.*, 1977). The frequency of multiple recombinants in *S. coelicolor* fusion products regenerated on non-selective medium was reported to be much higher than the one obtained by conjugation between mycelia. Recombinants were always observed in colonies arising from fused protoplasts and often the parental phenotypes were absent. It has been suggested that fragmentation of the parental genomes occurs after fusion and crossing-over between the fragments generate haploid recombinants (Hopwood and Wright, 1978, 1979).

Prototrophic selection after fusion between auxotrophic strains of *Streptomyces parvulus* or *Streptomyces antibioticus* resulted in the isolation of prototrophic recombinants in *S. parvulus*, while both prototrophic recombinants and unstable heterokaryons were formed by fusion in *S. antibioticus* (Ochi *et al.*, 1979).

A different approach was employed for selecting chromosomal recombinants in three species of Staphylococci. Fusion products between strains carrying plasmids with antibiotic resistance markers as well as chromosomal markers were directly selected by addition of different antibiotics to the medium. The selected clones carrying resistance plasmids from both parental strains displayed also chromosomal recombination. Both wild-type and double-defective recombinants were obtained at high frequency (Götz *et al.*, 1981).
2. **Cell fusion in filamentous fungi**

The first studies on protoplast fusion in fungi described spontaneous fusion between protoplasts of *Geotrichum candidum* and *Aspergillus nidulans*. Fusion at a low frequency was achieved by applying a centrifugal force or cold KCl to protoplasts (Ferenczy *et al*., 1974, 1975a). Similarly, fusion between protoplasts of *Phycomyces blakesleeanus* could be induced by seawater or Ca$^{2+}$ ions at high pH (Binding and Weber, 1974).

In all these studies complementation of auxotrophic requirements was used to select fusion products. These could be propagated on prototrophic medium indefinitely but auxotrophic segregants were readily formed on complete medium. Since loss of complementation of nutritional deficiencies always resulted in segregation of the parental types, it became evident from these early reports that heterokaryon formation was the most common consequence of protoplast fusion in these species.

With the introduction of fusion techniques which made use of PEG, the search for fusion products in fungal species was greatly facilitated (Ferenczy *et al*., 1975b, 1976; Anné and Peberdy, 1975). Many studies have reported successful protoplast fusion in several species. Analysis of fusion products in *Aspergillus flavus*, *A. niger*, *Penicillium frequentans*, *P. ramigena*, *P. chrysogenum*, *P. patulum*, *P. roquefortii* and *Mucor racemosus* (Ferenczy *et al*., 1976; Anné and Peberdy,
1976; Genther and Borgia, 1978) confirmed that fusion between fungal protoplast most frequently leads to heterokaryon formation. Diploids have been found to occur upon fusion at a very low frequency in *A. nidulans* (Ferenczy, 1976; Croft and Dales, 1979) and *P. chrysogenum* (Anné and Peberdy, 1976). Fusion of nuclei has also been observed in fused protoplasts of *Cephalosporium acremonium* by electron microscopy. However, the diploid state was transient since haploid recombinants could be recovered from crosses in which heterozygotes were not detected (Hamlyn and Ball, 1979).

Fusion between protoplasts of different species has been attempted in *Aspergillus* and in *Penicillium* species. Although complementation was reported to occur at much lower frequency in interspecific fusion experiments, hybrids could be isolated and analysed. Many unusual features were described for interspecific fusion products between the two distantly related species *A. nidulans* and *A. fumigatus* (Ferenczy, 1976; Ferenczy et al., 1977). For example, fusion products were neither heterokaryons nor diploids and under non-selective conditions only one of the parental types could be recovered. These observations suggested that fusion was followed by selective loss of chromosomes of one of the parental strains (Ferenczy et al., 1977).
Analysis of fusion products obtained between \textit{P. roquefortii} and \textit{P. chrysogenum} led to the characterisation of three types of hybrids, of which two could form only \textit{P. roquefortii} conidia but produced \textit{P. chrysogenum} penicillins and the third resembled the \textit{P. chrysogenum} parental type for both conidia morphology and penicillin production. These results have been interpreted as indicative of heterokaryon formation in type 1 and 2 and diploidy or, possibly, aneuploidy of type 3 hybrids (Anné \textit{et al.}, 1976).

Different results were reported for interspecific fusion between closely related species. Heterokaryons arose at high frequency upon fusion between \textit{A. nidulans} (Kevei and Peberdy, 1977), \textit{P. chrysogenum} and \textit{P. notatum} (Anné, and Peberdy, 1976), \textit{P. chrysogenum} and \textit{P. cyaneo-fulvum} (Peberdy \textit{et al.}, 1977).

Subsequent studies on distribution of segregants of hybrids isolated from heterokaryons between \textit{A. nidulans} and \textit{A. rugulosus} have shown that the exclusion of chromosomes during haploidisation was as random in these hybrids as in an intraspecific diploid. Hence the high degree of chromosome homology between the two species suggests that they are indeed very closely related (Kevei and Peberdy, 1979). Fusion between \textit{P. chrysogenum} and \textit{P. cyaneo-fulvum} has been reinterpreted recently as an example of intraspecific fusion from taxonomic considerations (Peberdy, 1979b).
More recently fusion techniques have been used for studying the significance of variation in mitochondrial genome size between members of the *A. nidulans* group. Comparison of restriction enzyme maps in *A. nidulans* and *A. nidulans* var. *echinulatus* revealed the presence of six insertions in the mt DNA of the latter species (Earl et al., 1981). Fusion products between these two species were selected using extrachromosomal antibiotic-resistance markers. The mt DNA from most of the hybrids thus obtained appeared to be like *A. nidulans* mt DNA but containing three of the *echinulatus* inserts. These were never lost in the presence of the *echinulatus* nuclear genome. Recombination of the three inserts into the *nidulans* mt DNA was shown to be selected by the *echinulatus* nuclear background. The other three inserts that could be lost have been compared to the optional introns described in *S. cerevisiae* mt DNA. When the same approach was adopted for analysing hybrids between *A. nidulans* and *A. nidulans* var. *quadrilineatus* similar results were obtained. The 'short' mt genome of *quadrilineatus* was never observed in the *A. nidulans* nuclear background unless it had recombined to acquire the 'extra DNA' present in the *nidulans* mt genome (Turner et al., 1981).

Undoubtedly the attainment of hybrids by protoplast fusion between otherwise incompatible species will prove very useful for providing some insight on the
interactions between the nuclear and the mitochondrial genomes in *A. nidulans* species.

3. **Cell fusion in yeast**

Shortly after the techniques for inducing protoplast fusion were established in bacteria and filamentous fungi, their application to yeast protoplasts was reported (Sipiczki and Ferenczy, 1977a; Van Solingen and Van der Plaat, 1977; Ferenczy and Maráz, 1977). Among these first reports the ones describing fusion between auxotrophic strains of *Candida tropicalis* revealed that, as in filamentous fungi, fusion in this yeast leads to heterokaryon formation (Fournier *et al.*, 1977; Vallin and Ferenczy, 1978). Under non-selective conditions heterokaryons were highly unstable and readily segregated the parental types. However, prolonged growth of fusion products in selective medium resulted in the isolation of stable prototrophs. These consisted of uninucleated cells which could be induced to segregate true recombinants and were therefore thought to be diploids (Fournier *et al.*, 1977). Heterokaryons could be easily distinguished from diploids (or aneuploids) when fusion products were selected from red (*ade*) and white (*aye*) mutants of *C. tropicalis*. Pink slow-growing heterokaryons, containing up to 8 nuclei, gave rise to white fast-growing prototrophs which contained only 1 nucleus. The DNA content per cell in these hybrids was
much higher than in the parental auxotrophs. However, the increase in DNA content was lower than the value expected in diploids, suggesting that the white prototrophs were aneuploids (Vallin and Ferenczy, 1978). Similarly, heterokaryons isolated by protoplast fusion between auxotrophic strains of *Candida albicans* could generate stable, rapidly growing prototrophs containing only 1 nucleus. Evaluation of DNA content per cell showed aneuploid DNA levels for most of the stable prototrophs analysed. It has been proposed that fusion of nuclei in heterokaryons would produce diploid nuclei which either stabilise as such or undergo partial haploidisation to various states of aneuploidy (Sarachek et al., 1981).

Heterokaryon formation, however, was not detected after protoplast fusion in most other yeast species. Diploidy is readily established upon fusion between *Sch. pombe* auxotrophs, even when the strains used have the same mating type (Sipiczki and Ferenczy, 1977a). Analysis of hybrids homozygous for mating type is usually carried out by inducing haploidisation with various agents, while fusion products heterozygous for mating type can sporulate (Maráz et al., 1978; Christensen, 1979).

The versatility of protoplast fusion for the production of diploids regardless of the mating ability of the two partners has been exploited in several yeast
species. In the life cycle of *Rhodosporidium toruloides*, for example, the haplophase is prevalent and stable diploids could only be obtained by fusing haploid protoplasts of identical mating type (Sipiczki and Ferenczy, 1977b). Genetic analysis of a mutation affecting the mating type locus in *Saccharomyces diastaticus* has been carried out by means of protoplast fusion (Takano and Arima, 1979) and a similar approach has been employed for studying sterile mutants of *Sch. pombe* (Thuriaux et al., 1980). Formation of hybrids in non-mating industrial strains has also been achieved by protoplast fusion thus providing a useful method for their improvement (Spencer et al., 1980; Hockney and Freeman, 1980).

However, the products of protoplast fusion are not always diploid as PEG induces aggregation between an indiscriminate number of protoplasts. With regard to this, a study on fusion between haploid protoplasts in a *Saccharomyces* species has reported the isolation of stable polyploids (Arima and Takano, 1979). Nevertheless an analysis of parameters such as cell volume, cell size and DNA content, as well as genetic characterisation of segregants, can easily establish the ploidy of fusion products.

Fusion techniques have also been applied to investigate yeast mitochondrial genetics. Transfer of mitochondria from a $p^+$ to a $p^-$ haploid has been
accomplished in \textit{S. cerevisiae} strains of the same mating type by constructing diploids by protoplast fusion. Haploids with the nuclear markers of the \(\rho^0\) strain but containing mitochondria from the other strain could be recovered by induced haploidisation of the fusion products (Ferenczy and Maráz, 1977, 1979; Gunge and Tamaru, 1978). More recently, comparisons on transmission of mitochondrial drug resistance markers in fusion products and diploids obtained by mating have been carried out also in \textit{S. cerevisiae} strains. The outcome of this analysis was that the frequencies of transmission and recombination of mitochondrial genes in fusion products were not affected either by homozygosis of the mating type or by the fusion process (Maráz and Šubik, 1981). Similar studies had been reported previously for \textit{Sch. pombe} (Lückermann \textit{et al.}, 1979).

A different approach, also aimed at transferring the mitochondrial genome to a different nuclear background, has been undertaken in \textit{S. cerevisiae} by fusing protoplasts with isolated mitochondria or with "mini-protoplasts". In the first case, mitochondria carrying an oligomycin resistance marker were fused with protoplasts from a \(\rho^0\) strain. \(oli^R\) respiratory-competent clones which had the nuclear genotype of the \(\rho^0\) strain were obtained at a low frequency (Gunge and Sakaguchi, 1979). The other method was based on the observation that during cell wall degradation small protoplasts are
released from buds which though still anucleate contain mitochondria. Their size enables them to be separated from normal protoplasts by low-speed centrifugation. They were called "mini-protoplasts" and used in fusion experiments with protoplasts from $p^0$ auxotrophic strains. Selection for respiratory-competent auxotrophs displaying the appropriate nuclear phenotype resulted in the isolation of the desired fusion products (Fukuda and Kimura, 1980). These methods, however, are applicable only to petite-positive yeasts. In order to study mitochondrial biogenesis in the petite-negative $K. lactis$ by exploiting fusion techniques, attempts to reproduce the same approach have been made by inducing transitory loss of mitochondrial DNA in one of the partners in fusion experiments. Unstable respiratory-deficient cells were induced with ethidium bromide in an auxotrophic mutant. These were transformed into protoplasts and fused with protoplasts from an untreated, respiratory-competent, mutant carrying complementing auxotrophic markers (Morgan et al., 1977). Application of this technique to characterising respiratory-deficient mutants in $K. lactis$ has allowed identification of the origin of two such mutations. Nuclear or mitochondrial inheritance of these mutations were discriminated on the grounds that respiratory competence can be restored in mitochondrially determined respiratory-deficient mutants by fusion only with a respiratory-competent strain but
not with an E.B.-induced respiratory-deficient strain (Allmark et al., 1978).

Intergeneric fusion of protoplasts with isolated mitochondria has also been reported. Protoplasts from a ρ⁻ mutant of *Saccharomyces cerevisiae* were fused with mitochondria isolated from *Hansenula wingei* or *Sch. pombe* and selected for respiratory competence. At a low frequency, respiratory-sufficient cells with the chromosomal genetic characteristics of *S. cerevisiae* were isolated. The successful transfer of mitochondria to the *S. cerevisiae* mutant was confirmed by analysis of absorption spectra which showed restoration of cytochromes *a* and *b* in fusion products (Yoshida, 1979).

To date only a few studies have analysed interspecific or intergeneric fusions in yeast. Stable hybrids have been obtained by fusing auxotrophic mutants of the closely related species *K. lactis* and *K. fragilis* (Whittaker and Leach, 1978). As revealed by cell size and DNA content estimations, most hybrids had lost chromosomes while one, which showed very high DNA content, was possibly a polyploid. Interestingly, all hybrids seemed to have lost the *K. lactis* mt DNA (buoyant density 1.692 g/cm³) and retained mt DNA of the same density as *K. fragilis* (1.683 g/cm³).

Protoplast fusion between auxotrophs of two *Schizosaccharomyces* species, *Sch. pombe* and *Sch. octoporus*, resulted in the isolation of prototrophic
hybrids. However, these could segregate only the *Sch. octopus* parental type. Furthermore, the hybrids exhibited osmotic sensitivity due to incomplete cell-wall formation and low viability (Sipiczki, 1979).

Intergeneric hybrids have been obtained by fusing mutants of *Candida tropicalis* and *Saccharomyces fibuligera*. Their characterisation, based on assimilation spectra of seven compounds for which the parental strains differed, led to a distinction of three groups of hybrids. The first two groups comprised hybrids that had most traits identical to one or the other parental species, while hybrids of the third group showed intermediate characters but were unstable. This instability was attributed to loss of chromosomes (Provost *et al.*, 1978).

Finally fusion between *S. cerevisiae* and *Sch. pombe* was attempted recently (Svoboda, 1980). After fusion colonies arose at a very low frequency and, moreover, their growth capacity was limited thus rendering impossible their characterisation.

**C. AIMS OF THE PRESENT STUDY**

As mentioned earlier, two hypotheses have been advanced to explain the *petite*-negative character of some yeasts. Either the structure of mtDNA or the enzymes
responsible for deletion are such that large deletions do not occur or else the physiological properties of the yeast precludes survival of petite mutants. To gain insight into this problem it was decided to examine whether mtDNA from a petite-negative yeast could undergo large deletions in the nuclear background of a petite-positive yeast.

Since *S. cerevisiae*, or any of the other known petite-positive species, does not interbreed with petite-negative species, cell fusion provides the only tool available at present for bringing together the nuclear genome of a petite-positive yeast with the mtDNA of a petite-negative species.

The possibility of applying cell fusion techniques to this end was analysed by pursuing in the first place fusion between a $\rho^0$ strain of *S. cerevisiae* and a $\rho^+$ strain of another petite-positive yeast. The search for a suitable petite-positive species, presenting both relatedness to *S. cerevisiae* and distinct properties which would facilitate the characterisation of interspecific hybrids, and for the experimental conditions for interspecific fusion is described in Chapter 1.

Finally, cell fusion between an auxotrophic strain of a petite-negative yeast and two different $\rho^0$ mutants of *S. cerevisiae* was attempted. Data pertaining to the characterisation of fusion products thus obtained are presented in Chapters 2 and 3.
CHAPTER 1

FUSION OF SACCHAROMYCES SPECIES
INTRODUCTION

One of the major problems concerning the characterisation of fusion products obtained between strains of different species is to establish the extent of genetic information retained from both parental strains when hybrids are selected for complementation of only a few markers. The genetic techniques used for analysing zygotes or intraspecific hybrids may not be always applicable to fusion products of distantly related species which are likely to be unable to undergo meiosis. Moreover, a detailed map of the nuclear genome has been determined only for \textit{S. cerevisiae} and \textit{S. pombe}, while for most of the other yeast species even the number of linkage groups is unknown.

A qualitative determination of nuclear DNA content in hybrids could, however, be possible if the nuclear DNA of the two parental species can be discriminated on the basis of its biochemical properties. It is known that the base composition of nuclear DNA varies considerably among yeast species (Meyer and Phaff, 1969; Bicknell and Douglas, 1970; Nakase and Komagata, 1971; Phaff \textit{et al.}, 1974; Yarrow and Nakase, 1975). Most species, including \textit{S. cerevisiae}, have been shown to have a GC content of 38-40\%, while a low 32-34\% GC value has been found for \textit{Saccharomyces unisporus}, \textit{Saccharomyces exiguus}, \textit{Saccharomyces telluris}, \textit{Pachytophaga transvaalensis} or, conversely, a high 45-48\% GC content for
Torulaspora globosa and Zygosaccharomyces mrakii. The composition of the nuclear genome in hybrids between *S. cerevisiae* and one of these species with low or high GC content could be easily evaluated by analytical determination of DNA buoyant densities in CsCl gradients.

In addition, valuable information on the expression of the parental genomes in such hybrids would be attained if the two parental species differ for several physiological characteristics, such as ability to utilise carbon compounds, sensitivity to some inhibitors of protein synthesis, growth at different temperatures and other parameters commonly used for taxonomic purposes (Provost *et al.*, 1978; Whittaker and Leach, 1978). Analysis of these characteristics in strains of species whose DNA base composition differs significantly from *S. cerevisiae* %GC value, was therefore carried out in order to select a strain suitable for fusion with *S. cerevisiae*.

After isolation of auxotrophic mutants from the chosen strain, the experimental approach to interspecific fusion was analysed by establishing first the conditions for intraspecific fusion between mutants with complementing markers.
MATERIALS AND METHODS

Strains, chemicals and the composition of the media are all described in the Appendix.

METHODS

Analysis of carbon compounds assimilation and cycloheximide resistance - A loopful of actively growing cells was resuspended in sterile water to give approximately $10^7$ cells/ml. A drop of the water suspension was placed on a set of NBA plates each containing 1% (w/v) of the carbon compound to be tested, except for raffinose which was used at a concentration of 2% (w/v) (Lodder, 1970). NBA medium containing 1% glucose and 100 µg/ml cycloheximide was inoculated in a similar way for testing cycloheximide resistance. As a control, a plate of NBA without any carbon compound and a GYP plate were also used. Plates were incubated at 25°C for 5-7 days.

Growth rate determination - Growth analyses were determined by following the change in optical density at 640 nm of cultures growing in 50 ml of 1% GYP in 125 ml conical flasks shaken at 200 rpm and maintained at 30°C in a water bath shaker. All optical density readings were taken on a Gilford 2400 spectrophotometer.
Isolation of auxotropic mutants - Cultures of S. unisporus to be mutagenised were grown overnight in GYP liquid medium and then 1 ml was added to 9 ml of water. The suspension was poured into a petri dish and exposed at 30 cm to a Philips shortwave, 15W lamp for 25 seconds. The irradiated culture was left to stand in the dark at room temperature for 60 min to avoid photoreactivation, then 1 ml of culture was diluted with 9 ml of fresh GYP and incubated for 3 hr at 30°C. A dilution of the culture was plated on indicator medium containing 0.03% (w/v) methylene blue and incubated at 30°C for 2-3 days.

Preparation and fusion of protoplasts - Exponentially growing cultures were harvested and washed twice with buffer (OSB) containing 0.15M citrate phosphate buffer (pH 5.4) and 0.6M KCl as osmotic support. After washing, cells were resuspended in OSB to give about $10^8$ cells/ml and treated with 0.2% (v/v) $\beta$-mercaptoethanol for 10 min at room temperature. Cells were washed again with OSB and incubated at 30°C in the presence of 1% (v/v) snail enzyme (Industrie Biologique Francaise) until their conversion into protoplasts was greater than 95%. This was measured by a decrease in absorbance at 640 nm of 1000-fold water diluted samples. Protoplasts from the two strains to be fused were washed twice with 0.4M CaCl$_2$ and mixed in a 1:1 ratio (about $10^8$
protoplasts of each auxotroph). The fusion mixture was centrifuged for 10 min and the pellet was gently resuspended in 2 ml of 35% polyethylene glycol (PEG MW4000, Sigma), in 50 mM CaCl₂. *S. unisporus* protoplasts were found to be susceptible to lysis at high concentrations of PEG, so a solution containing 20% PEG and 80 mg/ml dextran (MW 70,000, Pharmacia) was substituted. The mixed protoplasts were then incubated at 30°C for 20 min, washed with 0.4 CaCl₂ and plated on MM medium containing 0.6M KCl (MMK) at a density of about 10⁷ protoplasts per plate. Samples of protoplasts of each auxotroph, after undergoing the same treatment, were plated on MMK medium as a control for back-mutation and on MMK supplemented with the appropriate amino acids (MMKLA) for estimation of cell-wall regeneration. All plates were incubated at 30°C for 5 days.

**DNA estimation** - A modification of the procedure described by Burton (1956) and reported by Haber and Halvorson (1975) was followed. Briefly, about 2 x 10⁸ cells were harvested, washed with distilled water, and the pellets were frozen until used. After thawing, the pellets were resuspended in 8 ml of 0.2M perchloric acid (PCA) in 50% ethanol and left at room temperature for 40 min. Samples were sedimented at 5000 rpm for 10 min and washed with 4 ml PCA-ethanol. The pellets were then disrupted by mixing with a small amount of glass beads
and cooled in ice. After addition of 4 ml ethanol-ether (3:1, v/v), samples were incubated at 60°C for 5 min and centrifuged. The supernatant was carefully removed and the ethanol-ether step was repeated. The samples were sedimented and dried at 60°C. 1 ml of 1M PCA was added to each sample and incubated at 70°C for 3 min. After cooling, 1 ml of diphenylamine reagent (4% diphenylamine in glacial acetic acid containing 80 μg/ml acetaldehyde) was added. After incubation at 30°C for 17-19 hr, the samples were centrifuged, the supernatant collected and measured spectrophotometrically at 595 and 650 nm. Calf thymus DNA was used as standard. The number of cells employed was determined by counting samples with a haemocytometer prior to DNA extraction.

**X-ray irradiation** - Stationary-phase cultures of cells grown for 3-4 days in liquid GYP medium were diluted with sterile water to give approx. 5 x 10^3 cells per ml. 3ml aliquots were placed in petri dishes (4.5 cm dia.) and irradiated with X-rays obtained from a Siemens Stabilipan X-ray tube operated at 220 kV and 15 mA, the dose rate being 1.667 krad per min. After irradiation, cells were plated on GYP medium and incubated at 30°C for 5 days before colonies were scored.
Isolation of mitotic segregants from fusion products

Benlate treatment

Appropriate dilutions of cultures growing in GYP medium were spread on GYP agar plates containing 20 \( \mu \)g/ml benlate (Dupont, Paris) to give approximately 100 colonies per plate. After 5-7 days incubation at 30°C, sectoring colonies were subcloned on GYP agar medium. Single colonies from each subclone were tested on a set of MM agar plates where each plate was supplemented with a different combination of the requirements present in the two auxotrophs from which the fusion products were obtained.

Testing of 'small colonies'

Small colonies (about 1 mm dia) growing on GYP medium after X-ray irradiation of fusion products were tested in the same way as described for benlate-induced sectoring colonies.

RESULTS

A. Characterisation of species with different GC content from S. cerevisiae

Physiological characteristics of some species with either low or high GC content were analysed. The pattern of assimilation of a number of carbon compounds, resistance to 100 \( \mu \)g/ml cycloheximide and growth at 37°C
for six such species are summarised in Table 1-1. Three of these species were previously ascribed to the *Saccharomyces* genus and have been reclassified recently in three distinct genera (Barnett *et al*., 1979). It can be seen that, while *S. exigus* and *T. globoea* behave very similarly to *S. cerevisiae*, the other four species show differences with respect to *S. cerevisiae* for at least four characters. It should also be mentioned that *Z. mrakii* was found to grow poorly and only at temperatures not higher than 25°C.

Two of the species studied, *T. globoea* and *Z. mrakii*, were found to be petite-negative, while the remaining four species are petite-positive. However, there is a considerable variation in spontaneous petite frequency values among these species and with respect to *S. cerevisiae*. The lowest frequency has been reported for *P. transvaalensis* (0.003%). *S. unisporus*, *S. telluris* and *S. exigus* have similar petite frequencies of approximately 0.03% (Clark-Walker *et al*., 1981a), in contrast with *S. cerevisiae* which exhibits frequencies ranging from 0.6% to 9%, depending on the strain analysed (Oakley and Clark-Walker, 1978).

Considering these data, together with the results presented in Table 1-1, it is clear that *P. transvaalensis*, *S. unisporus* and *S. telluris* are the most suitable of the six species studied for fusion with *S. cerevisiae* in that they show several peculiar traits.
TABLE 1-1. Physiological characteristics of yeast species differing in DNA base composition from *S. cerevisiae*

<table>
<thead>
<tr>
<th>Species</th>
<th>D-Glucose</th>
<th>D-Galactose</th>
<th>Sucrose</th>
<th>Trehalose</th>
<th>Melibiose</th>
<th>D-Xylose</th>
<th>Raffinose</th>
<th>Maltose</th>
<th>Resistance to cycloheximide</th>
<th>Growth at 37°C</th>
<th>DNA base composition (° GC)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Saccharomyces cerevisiae</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>39-40.2&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Pachytrichospora transvaalensis</em></td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>32&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Saccharomyces uisporus</em></td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>32.4-32.8&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Saccharomyces telluris</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>32.8-34&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Saccharomyces exiguus</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>33.2-35.4&lt;sup&gt;b,c&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Torulaspora globosa</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>45.1-47.5&lt;sup&gt;b,e&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Zygosaccharomyces mrakii</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>48.5&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>


(+) growth; (-) no growth; (+) weak growth.
Fig. 1-1. Growth curves calculated using a polynomial regression. The curves represent:

- S. cerevisiae
- S. telluris
- S. unisporus
- P. transvaalensis
but also are likely to be more closely related to _S. cerevisiae_, as suggested by their **petite**-positive character.

An additional test was carried out on these three species to ensure that the division time of the chosen species and _S. cerevisiae_ were approximately the same since considerable discordance in behaviour during mitosis between the two fused partners could lead to rapid loss of one set of chromosomes. Fig. 1-1 demonstrates that the growth curves, and hence the division time, of _S. unisporus_ and _S. telluris_ are very similar to _S. cerevisiae_. _P. transvaalensis_, on the contrary, displays a division time 1.5 longer than the other three species. Consequently, _S. unisporus_ was selected as the species to be used for interspecific fusion with _S. cerevisiae_.

**B. Isolation of auxotrophic mutants of _S. unisporus_**

Ultraviolet radiation was used, as outlined in Methods, for inducing mutations to auxotrophy in _S. unisporus_ wild-type strain CBS398. Selection of auxotrophs was facilitated by employing the dye methylene blue in a similar way as for the phloxine B procedure described by Fink (1970). This latter compound could not be used since wild-type colonies of _S. unisporus_ readily became dark red in the presence of phloxine B. On the other hand, prototrophic colonies of _S. unisporus_
appear light blue when growing on medium containing methylene blue, thus rendering possible the identification of auxotrophs, which should become dark blue on the same medium once the nutrient they require for growth is exhausted.

Cells exposed to a dose of U.V. radiation which gave approximately 10% survival were therefore plated on indicator medium containing 0.03% methylene blue and incubated at 30°C for 3 days before dark blue colonies were scored. Screening of 3,297 colonies resulted in the identification of 182 dark blue putative mutants. A preliminary analysis of these colonies was carried out by testing them on MM, GlyYP and GYP plates since prototrophic petite colonies also become dark blue in the presence of the dye. Furthermore, blue mutants have been described in S. cerevisiae which, because of a change in cell wall or membrane structure, show greater uptake of methylene blue than wild-type (Mitchell and Bevan, 1973). The majority of 182 dark blue clones tested could be attributed to this type of mutant, as they grew well on all plates, while 39 were petites and only 12 clones did not grow on minimal medium, indicating that these were auxotrophic mutants. Analysis of the requirements present in the 12 mutants and their reversion frequencies to prototrophy revealed that 3 of the mutants had different requirements and also were stable auxotrophs, displaying a spontaneous reversion
frequency lower than $10^{-7}$. Two of these mutants (10, cys and 164, trp) were submitted to a further mutagenic treatment with U.V. radiation in order to induce a second auxotrophic mutation in each strain. Selection of double auxotrophs was achieved by using the same procedure applied for the isolation of single mutants, except for the addition of 50 μg/ml of the required amino acid (cysteine for mutant 10 or tryptophan for mutant 164) to the indicator medium containing methylene blue. Several double auxotrophs were isolated from both strains and some had reversion frequencies to the original requirement lower than $10^{-7}$. Two such mutants with complementing markers, 10.31 cys met and 164.16 trp his, were used in intraspecific fusion experiments.

C. Intraspecific protoplast fusion

(1) Fusion between S. cerevisiae auxotrophs. A ρ⁺ strain carrying the mutations ade8-18 lys2 (T) and the 500 eρ⁻ mutant (ade1, arg4), which lacks all detectable mtDNA, were used for formation of intraspecific hybrids by protoplast fusion. Strain T and 500eρ⁻ have the same mating type and therefore, all prototrophic colonies arising after PEG-induced fusion between their protoplasts should have resulted from fusion events rather than mating. The efficiency of cell-wall regeneration in protoplasts of both strains and the
TABLE 1-2. Efficiency of fusion

<table>
<thead>
<tr>
<th>Strain</th>
<th>% of regeneration</th>
<th>No. of prototrophs upon fusion</th>
<th>Frequency of prototrophs regenerated haploids*</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. cerevisiae T</td>
<td>6.5</td>
<td>35</td>
<td>8 x 10^{-5}</td>
</tr>
<tr>
<td>500 e^p^-</td>
<td>4.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. unisporus 164.16</td>
<td>3.3</td>
<td>19</td>
<td>1.1 x 10^{-6}</td>
</tr>
<tr>
<td>10.31</td>
<td>2.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Lowest haploid regeneration figure used in this ratio.
frequency of prototroph formation following fusion are presented in Table 1-2. The low frequency of regeneration displayed by both strains can probably be partly attributed to PEG treatment (Anne and Peberdy, 1976). The frequency of untreated protoplasts reverting to wall-bearing cells on regeneration medium was, in fact, 16.5% for strain T and 9.1% for 500 eρ−. In order to test if a lower concentration of PEG could improve regeneration without affecting fusion frequency, another fusion experiment was carried out between the same two strains. In this experiment, comparison of protoplast regeneration and fusion efficiency after PEG treatment at two different concentrations of the fusogen (20% and 35%) showed that there was no appreciable difference between the two PEG treatments. Lower concentrations were not tested since the stabilising effect of PEG would be lost below 20%.

To prove that prototrophic colonies isolated after protoplast fusion between strain T and 500 eρ− were indeed fusion products, a number of tests were undertaken on two ρ+ prototrophs, Sc.F10 and Sc.F33, chosen among the 35 colonies obtained from the first fusion experiment. It should be noted that only 2 of the 35 protoplasts, when tested for mitochondrial function, appeared to be respiratory-deficient. Analysis of DNA content per cell in Sc.F10 and Sc.F33 (Table 1-3) showed that Sc.F10 had the same amount of DNA per cell as the
Fig. 1-2. Dose-response curves to X-ray irradiation.

(a) *S. cerevisiae*: □ T; O F/T-3;
    ● Sc.F10; ●- Sc.F33.

(b) *S. unisporus*: □ 164.16; O Su.F1;
    ● Su.F2; ■ Su.F8.
diploid strain F/T-3, obtained by mating strain T with a strain of opposite mating type (F ade1 arg4) from which 500 ep− has been derived. However, Sc.F33 had only a small increase in DNA level over the haploid value, thus indicating that this isolate is probably aneuploid.

Another approach which can give information on the ploidy of fusion products is the analysis of their response to ionising radiation (Mortimer, 1958). As shown in Fig. 1-2a, both prototrophs have a considerably higher resistance to X-ray irradiation than the haploid parent. However, while the survival curve of Sc.FlO displays a shoulder characteristic of diploids, Sc.F33 had a linear response to the radiation. This finding strengthens the above mentioned observation on the aneuploid nature of Sc.F33. Comparison between the survival curve of the two fusion products and the diploid F/T-3 (Fig. 1-2a), also conforms to previous reports on increased sensitivity to X-ray radiation of diploids homozygous for mating type (Laskowski, 1962).

Finally, conclusive evidence that Sc.FlO and Sc.F33 are hybrids between strain T and 500 ep− was sought by analysing mitotic segregants recovered after treatment of the two fusion products with the haploidising agent benlate (Hastie, 1970). The effects of benlate on survival and production of sectored colonies form Sc.FlO and Sc.F33 are presented in Fig. 1-3a,b. The results in Table 1-4 show that segregants with recombined
<table>
<thead>
<tr>
<th>Strain</th>
<th>fg DNA/cell</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>S. cerevisiae</strong></td>
<td></td>
</tr>
<tr>
<td>T (haploid)</td>
<td>9.05 ± 0.13</td>
</tr>
<tr>
<td>F/T-3 (diploid)</td>
<td>18.74 ± 0.33</td>
</tr>
<tr>
<td>Sc.F10</td>
<td>18.66 ± 0.42</td>
</tr>
<tr>
<td>Sc.F33</td>
<td>10.75 ± 0.26</td>
</tr>
<tr>
<td><strong>S. unisporus</strong></td>
<td></td>
</tr>
<tr>
<td>164.16 (haploid)</td>
<td>8.69 ± 0.02</td>
</tr>
<tr>
<td>Su.F1</td>
<td>12.43 ± 0.20</td>
</tr>
<tr>
<td>Su.F2</td>
<td>13.07 ± 0.16</td>
</tr>
<tr>
<td>Su.F8</td>
<td>15.93 ± 0.30</td>
</tr>
</tbody>
</table>
Fig. 1-3a. Colony morphology and sectoring of *S. cerevisiae* fusion product Sc.F10 induced by benlate.
Fig. 1-3b. Survival curves and frequency of sectored colonies induced by benlate in *S. cerevisiae* fusion products. o Su.F10; x Sc.F33.

Fig. 1-4. Frequency of small colonies induced by X-ray irradiation in *S. unisporus* fusion products. o Su.F1; • Su.F2; ▲ Su.F8.
auxotrophic markers could be easily identified by testing small numbers of benlate-induced sectored colonies from both fusion products. Four of the mitotic segregants from Sc.F10 were also petite, while all segregants analysed from Sc.F33 were $\rho^+$. 

(2) Fusion between S. unisporus auxotrophs. PEG-induced fusion between protoplasts of the two double auxotrophs 10.31 and 164.16 previously isolated (Section B) followed by prototrophic selection was again the experimental approach adopted for isolation of hybrids in S. unisporus. Although at a lower frequency than in S. cerevisiae, prototrophic colonies were obtained from plating the fusion mixture on selective medium (Table 1-2), while no back-mutation was observed for either of the parental strains plated on the same medium. When the DNA per cell was estimated for three fusion products (Table 1-3), it was found that only one of them, Su.F8, displayed a nearly diploid value, while Su.F1 and Su.F2 showed an increase in DNA level over the haploid parent of approximately 50%. However, all three prototrophs were more resistant to X-ray radiation than the haploid parental strain 164.16 and their survival curve showed a non-linear response to the radiation (Fig. 1-2b). It can also be observed that the increase in resistance of S. unisporus fusion products is not as pronounced as for S. cerevisiae hybrids (Fig. 1-2a,b). This is probably
TABLE 1-4. Mitotic segregation of *S. cerevisiae* fusion products induced with benlate

<table>
<thead>
<tr>
<th>Fusion Products</th>
<th>Total colonies analysed</th>
<th>Prototrophic colonies</th>
<th>Auxotrophic colonies</th>
<th>adel</th>
<th>ade8-18</th>
<th>arg4</th>
<th>lys2</th>
<th>adel</th>
<th>ade8-18</th>
<th>adel</th>
<th>arg4</th>
<th>lys2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sc.F10</td>
<td>46</td>
<td>17</td>
<td>29</td>
<td>14</td>
<td>-</td>
<td>4</td>
<td>1</td>
<td>7</td>
<td>1</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sc.F33</td>
<td>15</td>
<td>9</td>
<td>6</td>
<td>2</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>1</td>
<td>-</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

TABLE 1-5. Mitotic segregation in "small colonies" produced after X-ray exposure of *S. unisporus* fusion products

<table>
<thead>
<tr>
<th>Fusion products</th>
<th>Total colonies</th>
<th>Prototrophic colonies</th>
<th>Auxotrophic colonies</th>
<th>his</th>
<th>met</th>
<th>cys met</th>
<th>his met</th>
<th>cys met</th>
<th>cys his</th>
<th>trp met</th>
<th>trp met</th>
</tr>
</thead>
<tbody>
<tr>
<td>Su.F1</td>
<td>22</td>
<td>15</td>
<td>7</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Su.F2</td>
<td>22</td>
<td>14</td>
<td>8</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Su.F8</td>
<td>21</td>
<td>12</td>
<td>9</td>
<td>3</td>
<td>-</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
due to the isogeneity of the two *S. unisporus* strains used for fusion.

Analysis of mitotic segregants from Su.Fl, Su.F2 and Su.F8 could not be carried out by inducing haploidisation with benlate, since these isolates were resistant to the drug at concentrations as high as 500 µg/ml. However, as it was observed that small colonies appeared among the survivors of X-ray treated fusion products (Fig. 1-4) and X-ray induced small colonies resulting from haploidisation had been described in *S. cerevisiae* diploids (Parry *et al.*, 1979), some of these small colonies from Su.Fl, Su.F2 and Su.F8 were analysed and found to be mitotic segregants. Recombinant phenotypes could be recovered from all three fusion products (Table 1-5).

D. *Interspecific fusion between S. cerevisiae and S. unisporus*

An ethidium bromide-induced petite isolated from *S. cerevisiae* strain T (*ade8-18, lys2*) was employed in fusion experiments with the *S. unisporus* auxotroph 164.16 (*trp, his*). Protoplasts from each strain were mixed and induced to fuse by the addition of a 20% PEG solution containing 50 mM CaCl₂ and 80 mg/ml dextran. As for intraspecific induced fusion, good aggregation between protoplasts, upon addition of PEG, was observed under the light microscope. Also in a similar manner to
intraspecific fusion experiments, fusion products were sought by prototrophic selection on MMK medium. The total number of protoplasts plated on MMK which could regenerate a cell wall, as calculated by the regeneration frequency on MMKA, was approximately $3 \times 10^7$. To ensure that conditions for selection of hybrids were as favourable as possible to sustain any possible interaction between the two genomes, selection was carried out at 25°C. However, even after 3 weeks incubation at 25°C, no colonies grew on selective medium. When the fusion experiment was repeated at different PEG concentrations (35%) or incubation conditions during PEG treatment (10-30 min at room temperature), prototrophic complementation was also absent. Only if the selective medium (MMK) was supplemented with 0.02% yeast extract were some microcolonies observed which proved to be inviable when transferred to GYP medium.

DISCUSSION

The well established techniques for intraspecific fusion in *S. cerevisiae* (Ferenczy and Maráz, 1977) also proved successful in this study for the isolation of fusion products between *S. unisporus* auxotrophs. The characterisation of a few of the prototrophs obtained via
protoplast fusion in both species clearly indicated that they were indeed fusion products which could produce segregants with recombinant as well as parental phenotypes. All but one (Sc.F33) also displayed a typical diploid response to X-ray irradiation (Laterjet and Ephrussi, 1949; Zirkle and Tobias, 1953; Mortimer, 1958). However, analysis of DNA content per cell showed that only one S. cerevisiae fusion product, and possibly one S. unisporus hybrid have the expected diploid DNA content. Other studies on intraspecific fusion in different species have reported similar frequent formation of aneuploids as a result of prototrophic selection after protoplast fusion between auxotrophs (Sipiczki and Ferenczy, 1977b; Whittaker and Leach, 1978) but it is not clear why it should be so.

Although the same experimental approach could be applied successfully for producing intraspecific hybrids in both S. cerevisiae and S. unisporus, formation of interspecific fusion products could not be attained. It cannot be excluded that one should expect a much lower frequency for production of hybrids between different species than in intraspecific fusion and, therefore, fusion between a very large number of protoplasts should be attempted. However, the finding that inviable microcolonies were formed when plates were supplemented with yeast extract, suggests that the two species are incompatible. Somewhat similar results have been
reported for fusion between strains of the two different genera *S. cerevisiae* and *Sch. pombe* (Svoboda, 1980). In a recent classification of yeasts (Barnett *et al.*, 1979) *S. unisporus* was retained in the *Saccharomyces* group, while *S. transvaalensis* and *S. kloekarianus* were reclassified in two separate genera. It is possible that *S. unisporus* may also be more distantly related to *S. cerevisiae* than thought at present.

Subsequently it was decided to proceed by attempting formation of hybrids through protoplast fusion between a petite-negative and a petite-positive yeast. Studies on fusion between an auxotrophic mutant of *K. lactis* and two different *S. cerevisiae* strains are described in Chapters 2 and 3.
CHAPTER 2

FUSION OF S. CEREVISIAE Δ6ep- WITH K. LACTIS
INTRODUCTION

Among the many petite-negative yeasts (DeDeken, 1961; Bulder, 1964; Lodder, 1970) *Sch. pombe* and *K. lactis* are the only species which have been frequently used in studies on both nuclear and mitochondrial genomes. While *Sch. pombe* is a fission yeast, vegetative reproduction in *K. lactis*, as in *S. cerevisiae*, is by budding. The two genera, *Kluyveromyces* and *Saccharomyces*, present also a few common physiological characteristics. Some *Kluyveromyces* species, including *K. lactis*, were in fact attributed to the *Saccharomyces* genus until van der Walt (1956) envisaged the genus *Kluyveromyces* to accommodate a newly isolated, budding, fermentative yeast. The separation between the two genera has been confirmed more recently by DNA-DNA hybridization studies (Bicknell and Douglas, 1970) and is, at present, well established (van der Walt, 1970; Barnett et al., 1979). *K. lactis*, was considered to be the most recently evolved species of *Kluyveromyces* since it represents the only exception to homothallism in the genus (van der Walt, 1970). Sexual reproduction in this yeast occurs between haploid heterothallic cells of opposite mating type in a manner analogous to the *S. cerevisiae* mating system. However, *K. lactis* and *S. cerevisiae* are not interfertile (Herman and Roman, 1966) and therefore the homology of the two
systems in respect to mating type is not known. Lack of interfertility is the reason why attempts at hybridisation between the two species may be approached only via protoplast fusion. Formation of prototrophs between complementing double auxotrophs of K. lactis and S. cerevisiae was therefore sought after inducing fusion between protoplasts of the two mutants. As it has been the aim of this study to investigate the possibility of analysing the behaviour of a mitochondrial genome from a petite-negative yeast in the presence of a petite-positive nuclear background, the S. cerevisiae strain herein employed was a ρ° petite generated by E.B. treatment of the D6 (arg8 met) auxotroph.

Analysis of fusion products between the two yeasts was also facilitated by the presence in K. lactis and S. cerevisiae of convenient distinct physiological characters. These differences reside in ability to assimilate maltose, sensitivity to cycloheximide and cryptopleurine, ability to grow at 37°C, splitting or arbutin, production of pulcherrimin and anaerobic growth (Van der Walt, 1970).

Finally, the application of DNA-DNA hybridization techniques to studying the nature of the genetic information in fusion products was investigated.
MATERIALS AND METHODS

Strains, chemicals and the composition of the media are described in the Appendix.

METHODS

Preparation and fusion of protoplasts

Protoplasts were isolated and fused as described in Chapter 1 Methods, except for a few modifications. Since the K. lactis strain used was very sensitive to the effect of β-mercaptoethanol, a solution containing 5% (v/v) snail enzyme and 1% driselase (Kyowa Hakko Kogyo Co. Ltd., Tokyo) in OSB buffer was adopted for producing protoplasts in this strain, without pretreatment with the reducing agent. Also, fusion was induced by incubation with PEG (35%, w/v) in 100 mM CaCl₂ for 30 min. at room temperature.

Analysis of physiological characters

Maltose assimilation and cycloheximide resistance — Actively growing cultures were tested as described in Chapter 1, Methods. All NBA plates were supplemented with the requirements of the strains to be tested.

Splitting of arbutin — A loopful of actively growing cultures was inoculated on slants prepared in the following manner. 5 ml aliquots of YP medium containing 2% agar were dispensed in 16 mm tubes and sterilised by
autoclaving. Immediately after sterilisation 0.5 ml of a 5% (w/v) arbutin solution and 2-3 drops of a 1% ferric ammonium citrate solution, both previously sterilised by passage through a Millipore filter, were added to each tube. The tubes were carefully shaken and slanted. After inoculation with the desired cultures, slants were incubated at 25°C for 5-7 days. Strains which hydrolyse arbutin developed a dark brown colour in this medium (Fig. 2-1).

Production of pulcherrimin - Formation of a red pigment, which diffuses into the medium, occurred in K. lactis strain 25, when growing on MM plates. However tests for pulcherrimin production were routinely performed by placing a drop of an actively growing culture on an MM plate previously spread with 0.1 ml of a sterile 5% solution of ferric ammonium citrate.

Sensitivity to cryptopleurine - Exponentially growing cells were dropped on GYP plates containing different concentrations of the drug. Ranges of drug concentrations used were: 0.1, 0.2, 0.5, 1.0 µg/ml. Plates were incubated at 25°C for 5-7 days.
Anaerobic growth determination - Actively growing cultures were streaked on GYP plates to which a solution of 30 mg ergosterol in 5 ml ethanol/litre and 5 ml/litre Tween 80 were added before autoclaving. A plate containing GYP medium not supplemented with ergosterol and Tween 80 was inoculated with the same cultures as a control. Under true anaerobic conditions none of the strains used should grow on GYP medium (Andreasen and Stier, 1953). All plates were incubated at 30°C under an atmosphere of hydrogen in a Gallenkamp anaerobic culture jar CX500 for 3 days.

DNA estimation, X-ray irradiation and benlate treatment

These methods were carried out following the procedures described in Chapter 1, Methods.

Isolation and purification of rDNA

Cultures were grown overnight at 30°C in 2 litres of GYP medium. Cells were harvested, resuspended in cold SE buffer (0.5M Sorbitol, 0.05M EDTA pH 7.0) and disrupted in a Braun homogeniser with glass beads. Unbroken cells and debris were removed by two low speed centrifugations. The supernatant was carefully decanted and centrifuged at 9,000 rpm for 20 min in a Sorvall SS34 rotor. The pellets were resuspended in 2% Sarkosyl before addition to a CsCl-bisbenzimide H33258 gradient (8.6g CsCl; 0.6 ml of 0.1M EDTA pH 7.0; 0.2 ml of a 1
mg/ml bisbenzimide solution; distilled water to give a volume of 7 ml). The gradient was adjusted to a final volume of 10 ml with water and centrifuged at 17,000 rpm, for 15 min. to remove the protein pellicle. The solution was then placed in a 50 Ti polyalomer tube and centrifuged at 44,000 rpm for 48 hr at 10°C. The rDNA was visualised by U.V. radiation (wave length = 350 nm) and removed by side-puncture using a 18-gauge needle. The dye was removed by 4 extractions with an equal volume of isopropanol saturated with 5M NaCl and the sample was dialysed against 0.1 x SSC. After dialysis, the solution was concentrated by rotary evaporation and extracted with equal volumes of phenol and chloroform. The DNA was precipitated by addition of 2.5 volumes of cold ethanol in the presence of 0.3M Na acetate (pH 5.5) at -20°C.

**Isolation of whole cell DNA**

Whole cell DNA was prepared from 5ml GYP stationary phase cultures as described by Davis *et al.*, 1980. Cells were harvested by centrifugation and washed with 1 ml 1M Sorbitol in a 1.5 ml eppendorf tube. After sedimentation, the pellet was resuspended in 0.5 ml of buffer (1M Sorbitol; 50 mM EDTA pH 8.5) containing 14 mM β-mercaptoethanol and 40 µg/ml zymolyase and incubated at 30°C for 30 min. Protoplasts were sedimented and lysed by the addition of 0.5 ml 0.2% SDS in 50 mM EDTA.
(pH 8.5). 1 μl of diethyl pyrocarbonate (Merck) was added and the suspension was incubated at 70°C for 15 min. SDS was precipitated by adding 50 μl of 5M K acetate and cooling the suspension in ice for at least 30 min. The precipitate was sedimented by centrifugation for 15 min. The supernatant was decanted into a new eppendorf tube and the DNA was precipitated by adding 2 volumes of ethanol at room temperature. After centrifugation for 15 sec., the precipitated DNA was dried and dissolved in 50-100 μl of 10 mM Tris pH 7.5, 1 mM EDTA pH 7.5., 1 μg/ml RNase A.

Isolation of plasmid DNA

Bacterial cells harbouring the desired plasmid DNA were grown at 37°C in 1 litre of L broth, containing the appropriate antibiotic for plasmid maintenance, to an OD₆₄₀ of 0.4-0.5. Chloramphenicol (50 μg/ml) was added and the culture was incubated overnight at 37°C for plasmid amplification. Cells were harvested by centrifugation at 5,000 rpm. Pellets were washed in TES buffer (50 mM Tris/HCl pH 8.0; 50 mM NaCl; 5 mM EDTA pH 8.0) and resuspended in 20 ml of 25% (w/v) sucrose in TES buffer. 2.5 ml of 0.25M EDTA (pH 9.0) and 10 mg lysozyme in 1 ml TES were added and the suspension was incubated on ice for 15 min. Spheroplasts were lysed by the addition of 1% Triton X-100 in TES and left on ice for a further 10 min. Plasmid DNA was separated from
chromosomal DNA and cellular debris by centrifugation at 18,000 rpm for 30 min at 0°C. Closed circular plasmid DNA was recovered by gradient centrifugation in CsCl-ethidium bromide (14 g CsCl, 4 mg E.B., in 10.5 ml of final volume) at 45,000 rpm for 48 hr at 10°C. Plasmid DNA was extracted by side-puncture, treated with isoamyl alcohol to remove E.B. and dialysed against 0.1 x SSC. The DNA was then precipitated with 2 volumes of ethanol at -20°C.

Restriction endonuclease digestion of DNA and electrophoretic separation of fragments

Purified DNA (0.5-10 μg) was digested in 20 μl of an appropriate buffer to which 2-4 units of enzyme were added. The mixture was incubated at 37°C (BamHI, EcoRI) or 25°C (SmaI) for 1-2 hr. Digestion was stopped by the addition of 5 μl of 0.1% bromophenol blue in 50% (w/v) sucrose and 0.6% (v/v) Sarkosyl. The following buffers were used. BamI: 6 mM Tris/HCl pH 7.5, 6 mM MgCl₂, 50 mM NaCl, 6 mM β-mercaptoethanol; EcoRI: 50 mM Tris/HCl pH 7.5, 100 mM NaCl, 10 mM MgCl₂; SmaI: 15 mM Tris/HCl pH 8.5, 15 mM NaCl, 6 mM MgCl₂.

Fragments were separated electrophoretically in a flat bed apparatus (20 x 20 x 0.4 cm) using 1% agarose containing 1 μg/ml E.B. and in the presence of 40 mM Tris, 20 mM Na acetate and 1 mM EDTA adjusted to pH 7.8 with acetic acid. Electrophoresis was at 35 mA for 16 hr.
Transfer of DNA to nitrocellulose filters

DNA fragments separated by electrophoresis were bidirectionally transferred by gel blotting (Smith and Summers, 1980). Samples to be transferred from an agarose slab gel were denatured in situ in three steps, each of duration of 30 min, by placing the slab in three different solutions. These were: (a) 0.5N NaOH, 0.5M NaCl; (b) 0.5M Tris pH 7.5, 2M NaCl; (c) 20 x SSC (0.15M NaCl, 0.015M Tri sodium citrate).

Bidirectional blotting was carried out by placing the slab between two nitrocellulose filters (Millipore) previously cut to size and soaked in 20 x SSC for 10 min. After 1-2 hr the filters were removed, soaked in 2 x SSC for 10 min and dried in a vacuum oven at 80°C for 1½ hr.

Labelling of DNA

DNA samples to be labelled were cleaved by restriction nucleases as follows: rDNA with SmaI, Yep6 and X-4 DNA with BamHI, pYel61A3 DNA with EcoRI.

The DNA was labelled to high specific activity by using random primers and the Klenow fragment of DNA polymerase I according to a procedure adapted from Goulian et al. (1973). A random primer mixture (50 μg), prepared from calf thymus DNA, was added to the DNA (0.5 to 2 μg) and the volume adjusted to 20 μl. This mixture was denatured by heating to 90°C for 3 min followed by
rapid cooling in ice. For chain elongation the
denatured DNA mixture was made to 50 μl and contained
final concentrations of 20 mM Tris HCl (pH 7.4), 10 mM
MgCl₂, 5 mM mercaptoethanol, 1 mM of dCTP, dGTP and TTP,
50 μCi of [³²P]dATP (>2000 Ci/mmol) and 3 units of DNA
polymerase I. Incubation was at 37°C for 1 h and the
reaction was terminated by extraction with phenol/CHCl₃
before separation of the labelled DNA from the
[³²P]mononucleotide by chromatography on G50 Sephadex.

**DNA-DNA hybridisation**

Nitrocellulose filters were sealed in plastic bags
to which were added 2-4 ml of 3 x SSC containing 0.2% (w/v) bovine serum albumin (Sigma), 0.2% (w/v) Ficoll (Pharmacia), 0.2% (w/v) polyvinyl pyrolidone PVP360 (Sigma), 0.1% (w/v) SDS (Sigma), 10 mM HEPES pH 7.0, 18 μg/ml calf thymus DNA and 10 μg/ml *E. coli* tRNA. The filters were preincubated at 65°C in a water bath for 2 hr before addition of ³²P-labelled DNA (2-5 x 10⁶ cpm). After incubation for 20 hr, the filters were washed in 1 x SSC for 3 hr at 37°C, dried by blotting and subjected to autoradiography at -80°C with Kodak X-Omat XS5 X-ray film using an intensifying screen (Ilford).
Mating and ascus dissection

Haploids of opposite mating type were grown overnight in liquid GYP, 0.5 ml of each strain were mixed and 1 ml of fresh GYP containing 10% glucose was added to the culture. The mating mixture was incubated for 3 hr, with shaking at 30°C, washed once with sterile water and spread on MM plates. Plates were incubated at 30°C for 3-5 days before diploids were picked, resuspended in water and dropped on malt agar plates. These were left at room temperature for 3-5 days before asci were observed. The sporulated culture was treated with snail enzyme for 5 min to digest the ascus wall and asci were dissected with a de Fonbrune pneumatic micromanipulator. Dissected ascospores were germinated on solid GYP at 30°C.

RESULTS

A. Isolation of fusion products between D6ep and K. lactis

Fusion between protoplasts of the S. cerevisiae strain D6ep (arg8 met) and protoplasts of a K. lactis mutant, K25, requiring lysine and uracil for growth was promoted by PEG as intensive agglutination could be observed during incubation of the protoplasts in the presence of the fusogen. Examination of the fusion
mixture under the light microscope revealed that most protoplasts were involved in the formation of aggregates, some comprised of as many as 10-15 protoplasts. After 20-30 min of PEG treatment, several very large protoplasts could be seen which probably had resulted from complete fusion between a few protoplasts.

Two different approaches were adopted for selecting fusion products. Prototrophic selection on MMK medium was employed as well as selection for cytoductants on GlyMMK (MMK medium containing only 0.1% glucose and 4% glycerol) supplemented with 50 \( \mu g/ml \) arginine and 50 \( \mu g/ml \) methionine. In the latter case only protoplasts from the D6ep\(^-\) strain that had acquired mitochondria from K25 during fusion could regenerate and produce colonies. However, after 2 weeks incubation at 25°C, a few colonies grew on MMK but none on GlyMMK + arg met. The total number of regenerated protoplasts, as calculated by the number of protoplasts which gave rise to colonies on MMKA medium, that were used for prototrophic selection of fusion products was approximately 5 x 10\(^7\). Fourteen colonies were isolated on MMK plates. When these were subcloned on MM and GYP, none grew on MM and all gave mixed colonies on GYP which had either the apiculate morphology typical of \( S. \) \textit{cerevisiae} or the flat appearance characteristic of \( K. \) \textit{lactis}. A single colony of each type from each subclone was tested on a set of MM plates supplemented with all
possible combinations of the requirements present in the two parental strains. All but four colonies had the auxotrophic markers of either D6ep⁻ or K25. The four isolates, each from independently isolated fusion products, were now requiring lysine, uracil and arginine for growth.

This unusual result was reproduced when the same two mutants, D6ep⁻ and K25, were fused again under the same conditions. In this second experiment 18 colonies grew on MMK and these were tested immediately on a set of MM plates of similar composition to the ones employed for the second subcloning in the previous experiment. Three clones had the phenotype arg met, one was lys ura and fourteen were lys ura arg. It is relevant that direct subcloning on MM containing lysine, uracil and arginine gave a higher number of isolates with the lys ura arg phenotype. Presumably, if the same procedure for screening colonies appearing on MMK were applied in the first experiment more clones of this phenotype should also have been isolated.

Subsequently, to verify if this was a peculiar property of the arg8 gene of S. cerevisiae, K25 was fused with two different mutants of S. cerevisiae carrying mutations at the arg8 locus. However, fusion between K25 and strain X1049-9c (arg8 trp1 ura3 his6 asp5) or strain JB82 (arg8 ade2-2 gal2) did not give rise to colonies on MMK, although the number of regenerated
protoplasts employed was comparable to that used in fusion experiments with D6ep-.

The four fusion products isolated in the first fusion experiment between K25 and D6ep- (KD3, KD11, KD13 and KD14) were submitted to further analysis.

B. Characterisation of fusion products between K25 and D6ep-

(1) Analysis of physiological characters. Some of the peculiar traits which separate Kluyveromyces species from Saccharomyces and also characterise K25 in respect to D6ep- are: assimilation of maltose, resistance to 100 μg/ml cycloheximide, absence of growth under anaerobic conditions and at 37°C. Furthermore, K. lactis can hydrolyse β-glucosides, such as arbutin. This trait is easily detected since splitting of arbutin produces hydroxyquinone which will give a brown colour with any soluble ferric salts (Fig. 2-1). K. lactis also produces a red pigment related to, or identical to, pulcherrimin (Lodder, 1970; Wickerham and Burton, 1956). These properties were tested in the fusion products KD3, KD11, KD13 and KD14 and the results are presented in Table 2-1. Except for lack of maltose assimilation and slower production of pulcherrimin, all fusion products showed the same characteristics as K25. Similarly, when sensitivity to another inhibitor of protein synthesis, cryptopleurine (Grant et al.,
Fig. 2-1. Splitting of arbutin in medium containing ferric salts. (A) *S. cerevisiae* D6; (B) *K. lactis* 25.
<table>
<thead>
<tr>
<th></th>
<th>K25</th>
<th>D6eρ^-</th>
<th>KD3</th>
<th>KD11</th>
<th>KD13</th>
<th>KD14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maltose assimilation</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cycloheximide resistance</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Splitting of arbutin</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Pulcherrimin production</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Anaerobic growth</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Growth at 37°C</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
1974), was analysed the fusion products had the same level of sensitivity as K25 (Table 2-2).

(2) Analysis of DNA content and mitotic segregation. Estimation of DNA content per cell showed that DNA levels in fusion products were not significantly different from values obtained for K25 and D6ep− (Table 2-3). This implied that the fusion products were either haploid, or near to haploid. To verify this, KD3, KD11, KD13 and KD14 were submitted to X-ray irradiation and the dose-response curves are given in Fig. 2-2. All fusion products were slightly more sensitive than K25 and, except KD3, presented a linear response to the radiation. Moreover, no small colonies with different phenotype from the fusion products (lys ure arg) could be isolated. Mitotic segregants were also sought by exposing the fusion products to 200 μg/ml benlate. At this concentration of the drug KD3, KD11, KD13 and KD14, like the \textit{K. lactis} strain from which they were derived, exhibited 35-40% survival. However, all fusion products behaved like the haploid parent K25, that is, no segregants could be identified. These results confirm the data on the haploid nature of the fusion products obtained by estimation of DNA content. Further evidence on the general absence of \textit{S. cerevisiae} chromosomes was sought by DNA-DNA hybridisation analysis using \textsuperscript{32}P-labelled rDNA and the \textit{S. cerevisiae his3} sequence as
### TABLE 2-2. Sensitivity to cryptopleurine

<table>
<thead>
<tr>
<th>Cryptopleurine (μg/ml)</th>
<th>0.1</th>
<th>0.2</th>
<th>0.5</th>
<th>1.0</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strain:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K25</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D6eρ^-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>KD3</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>KD11</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>KD13</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>KD14</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

### TABLE 2-3. DNA content per cell

<table>
<thead>
<tr>
<th>Strain</th>
<th>fg DNA/cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>K25</td>
<td>7.250 ± 0.7</td>
</tr>
<tr>
<td>D6eρ^-</td>
<td>8.223 ± 1.6</td>
</tr>
<tr>
<td>KD3</td>
<td>7.337 ± 0.7</td>
</tr>
<tr>
<td>KD11</td>
<td>8.209 ± 1.4</td>
</tr>
<tr>
<td>KD13</td>
<td>8.588 ± 1.5</td>
</tr>
<tr>
<td>KD14</td>
<td>8.333 ± 0.8</td>
</tr>
</tbody>
</table>
Fig. 2-2. Dose-response curves to X-ray irradiation.

The curves represent: ● K25; X D6ep−; ○ KD3;
□ KD11; ■ KD13; △ KD14.
probes.

(3) **Analysis of specific DNA sequences in fusion products by molecular probing.** The ribosomal DNA of *S. unisporus* has higher buoyant density (1,704 g/cm$^3$) than the rest of the nuclear DNA (1,692 g/cm$^3$). This property allows good separation of the rDNA from the nuclear DNA by CsCl-bisbenzimide density gradient centrifugation. The difference in buoyant density between rDNA and nuclear DNA in *S. cerevisiae* is not as pronounced and therefore contamination of rDNA with nuclear DNA cannot be easily avoided. On the other hand, *S. unisporus* rDNA devoid of any nuclear DNA contamination can be isolated in a single step. A sufficient degree of sequence homology between rDNA cistrons in different yeasts made possible the analysis of the organisation of rDNA in the fusion products obtained between K25 and D6ep$^-$ by using *S. unisporus* rDNA, labelled with $^{32}$P, as a probe. Total DNA isolated from K25, D6ep$^-$, KD3, KD11 and KD14 was restricted with *BamHI*. The fragments generated by restriction were electrophoretically separated, transferred to nitrocellulose and hybridised to $^{32}$P rDNA from *S. unisporus* (Fig. 2-3). The K25 DNA sample was only partially digested with *BamHI* to verify that the high molecular weight fragments hybridising to the probe in KD3, KD14 and, to a smaller extent, KD11 DNA,
Fig. 2-3. Hybridisation patterns of BamHI digested total DNA to $^{32}$P labelled rDNA.
(a) K25; (b) KD3; (c) KD11; (d) KD13; (e) KD14; (f) D6ep$^-$. 
represent incomplete digestion of these samples rather than the presence of rDNA from D6ep-'. Complete digestion with *BamHI* of K25 in fact produces only one fragment corresponding to the lower band in Fig. 2-3. It can be concluded that all fusion products retained only *K. lactis* rDNA.

Since the *arg8* locus in *S. cerevisiae* is situated on the left arm of chromosome XV (Hilger and Mortimer, 1980), retention of this chromosome in fusion products could be detected by DNA-DNA hybridisation with labelled plasmid DNA containing the *his3* sequence from *S. cerevisiae* as this gene maps on the right arm of the same chromosome (Mortimer and Hawthorne, 1966; Culbertson *et al.*, 1980). Two plasmids, both containing the *his3* sequence, were available: YEp6 and X-4. The former has been prepared and characterised by Struhl *et al.* (1979) and contains part of the 2 μm DNA of *S. cerevisiae* as well as the *his3* gene. The plasmid X-4 lacks 2 μm DNA as it was formed by ligation of the 1750 bp *BamHI* fragment containing the *his3* gene into the *BamHI* site of pBR322.

Hybridization of *EcoRI* digested total DNA from the fusion products to labelled YEp6 or X-4 DNA was not detected (Fig. 2-4). Hence neither the *his3* locus nor the 2 μm DNA from *S. cerevisiae* are present in KD3, KD11, KD13 and KD14.

As fusion products with the *arg* phenotype could not be isolated between K25 and two other *arg8* mutants of *S.*
Fig. 2-4. Hybridisation patterns of *EcoRI* digested total DNA to $^{32}$P labelled YEp6 (A) and X-4 (B).

(a) K25; (b) KD3; (c) KD11;
(d) KD13; (e) KD14; (f) D6ep−.
*cerevisiae* (see Section A of this Chapter), a singular type of mutation could be present at the *arg8* locus of D6ep−, for example, a mutation produced by transposition of a repetitive DNA sequence similar or identical to the Ty elements recently described by Cameron *et al.* (1979). Such a transposable sequence could be responsible for the *arg* phenotype of the fusion products. Alternatively, this might have resulted from translocation of part of the distal end of chromosome XV of *S. cerevisiae* to a *K. lactis* chromosome. The former hypothesis was tested by hybridising *EcoRI* cleaved total DNA from the fusion products to plasmid DNA carrying the Tyl-161 (pYel61A3) sequence (Kingsman *et al.*, 1981). The results are presented in Fig. 2-5. Two points should be observed. Firstly, 5 fragments of K25 total DNA hybridised to the Tyl-161 sequence, indicating that a repetitive sequence at least partially homologous to the Tyl element of *S. cerevisiae* is also present in *K. lactis*. Secondly, total DNA from the fusion products showed hybridisation at six fragments. The largest of these fragments was not observed in K25 and could correspond to one of the bands found in D6ep−. Finally, genetic analysis of the fusion products was undertaken in order to investigate if chromosomal abnormalities could be detected.
Fig. 2-5. Hybridisation patterns of *EcoRI* digested total DNA to $^{32}\text{P}$ labelled pYel61A3.

(a) K25; (b) KD3; (c) KD11; (d) KD13; (e) KD14; (f) D6ep$^-$.
(4) Genetic analysis of fusion products. All fusion products could mate with a strain of *K. lactis*, WM52 *ade his* (Tingle *et al.*, 1968), of opposite mating type to K25. Sporulation was induced in diploids obtained by mating WM52 with KD14 and 67 four-spored asci were dissected. Of these, only 26 gave rise to 4 viable spores (Fig. 2-6b) which could be analysed in respect to the *arg* marker. As shown in Table 2-4, the *arg* phenotype segregated in a 2:2 fashion. The same pattern of segregation was observed for all the other phenotypes, except for *ade*, which is likely to be due to a double mutation. Linkage relationships between markers can be deduced from the data presented in Table 2-5. Absence of nonparental ditype (NPD) asci for *arg/his* and *lys/ura* segregants indicates that the *arg* mutation in KD14 is linked to the *his* locus and also the *lys* and *ura* markers are linked.

When the viability of ascospores was examined, it was observed that the largest class of the dissected asci contained only 3 viable spores (Fig. 2-6b), while tetrads from a cross between WM52 and K25 did not show any abnormality in respect to the same character (Fig. 2-6a). This could be due to an inversion (Perkins and Barry, 1977) that may have occurred during transposition of a *S. cerevisiae* chromosomal segment to a *K. lactis* chromosome or, alternatively a different gene order could be present in the two yeasts due to the transposed
### TABLE 2-4. Segregation of auxotrophic markers in asci from cross WM52×KD14

<table>
<thead>
<tr>
<th>Segregation in asci +:-</th>
<th>ade</th>
<th>his</th>
<th>lys</th>
<th>ura</th>
<th>arg</th>
</tr>
</thead>
<tbody>
<tr>
<td>0:4</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1:3</td>
<td>15</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>2:2</td>
<td>10</td>
<td>25</td>
<td>26</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>3:1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

### TABLE 2-5. Analysis of linkage between markers in asci from cross WM52×KD14

<table>
<thead>
<tr>
<th></th>
<th>PD*</th>
<th>NPD*</th>
<th>TT*</th>
<th>Total No. of tetrads</th>
</tr>
</thead>
<tbody>
<tr>
<td>arg/his</td>
<td>15</td>
<td>0</td>
<td>10</td>
<td>25</td>
</tr>
<tr>
<td>arg/lys</td>
<td>4</td>
<td>9</td>
<td>12</td>
<td>25</td>
</tr>
<tr>
<td>arg/ura</td>
<td>6</td>
<td>12</td>
<td>6</td>
<td>24</td>
</tr>
<tr>
<td>lys/ura</td>
<td>12</td>
<td>0</td>
<td>14</td>
<td>26</td>
</tr>
<tr>
<td>his/lys</td>
<td>2</td>
<td>11</td>
<td>12</td>
<td>25</td>
</tr>
<tr>
<td>his/ura</td>
<td>5</td>
<td>8</td>
<td>11</td>
<td>24</td>
</tr>
</tbody>
</table>

*PD, parental ditype;  NPD, nonparental ditype and TT, tetratype asci.
Fig. 2-6. Viability of ascospores in dissected asci.

(a) Tetrads from cross WM52 x K25.

(b) Tetrads from cross WM52 x KD14.
Fig. 2-7. The origin and constitution of asci containing various numbers of inviable spores from crosses of Normal (white centromeres) x Inversion (black centromeres). Chromosomal segments with deficiencies are represented as dotted lines.
segment. A model of the possible events leading to spore inviability, during pairing of a normal chromosome with a chromosome carrying an inversion, is given in Fig. 2-7.

This model predicts a higher frequency of the 3:1 viable:inviable ascospores than the model presented by Perkins & Barry (1977) which deals with a pericentric chromosomal inversion. Thus a model whereby a single transposition or an inversion exclusive of the centromere is more in accord with the observed data.

DISCUSSION

Transient complementation of auxotrophic requirements was obtained after inducing fusion between protoplasts of \( K. \) \( \text{lactis} \) (\( \text{lys} \ \text{ura} \)) and \( S. \ \text{cerevisiae} \ (\text{arg8 met}) \). Growth of the transient prototrophs on selective medium into sizable colonies was possibly promoted by cross feeding within the colonies.

The unstable prototrophs segregated with the unexpected phenotype \( \text{lys} \ \text{ura} \ \text{arg} \). From their physiological characteristics, which with the exception of maltose assimilation are always similar to those of the \( K. \ \text{lactis} \) strain, and also from DNA content, it was deduced that these fusion products were haploid or close to haploid. Absence of maltose assimilation in the
fusion products is not of easy interpretation, since expression of this trait in yeast is due to a polymeric gene system (Mortimer and Hawthorne, 1969) controlled by a complex regulatory mechanism (Evans and Wilkie, 1976). It is also not clear why, although more segregants with the D6ep\(^{-}\) phenotype (arg met) resulted from the unstable prototrophs than K25-like colonies (lys ura), there seems to be a "dominance" of \(K.\ lactis\) in \(lys ura\) arg products from the initial prototrophs.

With regard to the haploid nature of these fusion products, additional evidence was inferred from lack of mitotic segregation in fusion products treated with ionising radiation or benlate. Moreover, the absence of \(S.\ cerevisiae\) rDNA and \(his3\) sequences in these isolates suggests that the arg phenotype had resulted from interactions between small portions of the parental genomes. It is interesting also that no hybridization was found between the \(his3\) sequence of \(S.\ cerevisiae\) and \(K.\ lactis\) DNA. This was observed even at lower stringency conditions (incubation at 62°C), thus indicating that there is small homology between the \(his3\) genes of the two yeasts.

Interesting properties have been described for repetitious DNA sequences which affect gene expression in \(S.\ cerevisiae\). Dispersed repetitive DNA sequences in the genome of \(S.\ cerevisiae\) were first discovered by Cameron et al. (1979) during studies on the \(sup4\) region.
of chromosome X. A fragment of this region cloned in phage λ was found to hybridise to about 35 different EcoRI fragments of yeast DNA. This family of repeated DNA sequences was called Tyl. A Tyl element is a 5.6 kb sequence comprising a direct repeat of 0.3 kb in length at each end. These terminal repeats, called J and also present in multiple copies, were not always associated with Tyl, as there are at least 100 J sequences per haploid genome. Alterations both in sequence composition and genomic location of Tyl units have been observed between different strains of S. cerevisiae and also during prolonged cultures of the same strain. It followed from this that Tyl sequences were postulated to be transposable elements.

More evidence in support of this hypothesis was given by the finding that a mutation at the his4 locus (his4-912) was due to insertion of a Tyl sequence (Ty912) in the promoter region of the gene. His+ revertants of the his4-912 mutant had a number of chromosomal aberrations (i.e. deletions, translocations, inversions) which resulted from recombinational events between Tyl or J sequences (Chaleff and Fink, 1980; Roeder and Fink, 1980). Analysis at the DNA sequence level of the insertion process of a Tyl element at the his4 locus revealed that a 5 bp duplication was generated in the target DNA at each end of the Tyl inserted sequence (Farabaugh and Fink, 1980; Gafner and Philippsen,
This resembles what is found for bacterial transposons, which produce duplication of 9 bases (IS1, Tn5, Tn9, Tn10) or 5 bases (IS2, Tn3) at the insertion site (Grindley and Sherratt, 1978).

Subsequently, two new classes of dispersed repetitive sequences from *S. cerevisiae* were described, Tyl-17 and Tyl-161 (Kingsman *et al*., 1981). Both are structurally related to the Tyl element. Tyl-17, like Tyl, is flanked by *ø* elements and its length is 5.6 kb but two large regions (approx. 50% of the total length) of the Tyl-17 sequence are not homologous to Tyl. Tyl-17 was isolated from the *leu2* region of chromosome III and is present in six copies per haploid genome. The other element, Tyl-161, was found near the *PGK* locus, also on chromosome III, and it is a Tyl sequence with a 1.2 kb insertion. Studies on constitutive expression of alcohol dehydrogenase II in *S. cerevisiae* have also led to the discovery that most of the ADHII constitutive mutants investigated resulted from insertion of a sequence of the Tyl family near the 5' end of the structural gene (*ADR2*). Restriction enzyme analysis of the inserted elements showed that in all but one case they appear to be identical to Tyl, while in one mutant the element is very similar to Tyl-17 (Williamson *et al*., 1981; Ciriacy and Williamson, 1981).

The mechanism by which these elements can transpose
to different loci is probably similar to that of prokariotic transposons (Grindley and Sherratt, 1978), although Tyl sequences promote chromosomal aberrations that are not observed for bacterial transposons (Roeder and Fink, 1980; Chaleff and Fink, 1980).

Transposition of a similar, or unrelated, sequence from the \( \text{arg8} \) locus of \( D6\text{ep}^- \) to an homologous segment of a \( \text{K. lactis} \) 25 chromosome could have occurred in fused cells before loss of \( S. \text{cerevisiae} \) chromosomes. However, this hypothesis could be substantiated only by analysis at the molecular level of the \( \text{arg} \) locus in \( D6\text{ep}^- \) and in the fusion products (i.e. by cloning the \( \text{arg8} \) gene of \( S. \text{cerevisiae} \)).

Examination of spore viability in crosses between a normal \( \text{K. lactis} \) strain and a fusion product gives strong evidence that chromosomal aberrations are present in the fusion product (Perkins and Barry, 1977). These may have arisen from recombinational events between partially homologous segments of a \( \text{K. lactis} \) and \( S. \text{cerevisiae} \) chromosome, thus generating the \( \text{arg} \) phenotype. Again, this possibility can be examined only by studying the molecular structure of the \( \text{arg} \) locus in fusion products.
CHAPTER 3

FUSION BETWEEN S. CEREVISIAE Fep\textsuperscript{−} AND K. LACTIS
INTRODUCTION

During the previous study on fusion between a *S. cerevisiae* strain carrying an *arg8* mutation and the *K. lactis* auxotroph K25 (Chapter 2) only unstable prototrophs were obtained. These segregated amongst other products the *K. lactis* phenotype with the addition of a requirement for arginine. Since it was of interest to test if different mutations to arginine auxotrophy in the *S. cerevisiae* fusion partner behaved in a similar manner, attempts were made to reproduce the same results with two different *arg8* mutants as reported in Chapter 2. Also directed to the same analysis, fusion between the same *K. lactis* strain and a strain of *S. cerevisiae* carrying an *arg4* mutation (*F ade1 arg4-16*) was undertaken and, for the same reasons that required the use of a ρ° mutant of D6, an ethidium bromide-induced petite of strain F was employed.

Unlike D6ep⁻, Fep⁻ gave rise to stable prototrophs upon fusion with *K. lactis* 25. The characterisation of these prototrophic isolates and the analysis of their response to petite induction are described in this Chapter.

MATERIALS AND METHODS

Strains, chemicals and composition of media are given in the Appendix.
METHODS

Preparation and fusion of protoplasts. Protoplasts were isolated and fused following the modifications described in Chapter 2, Methods, of the procedure given in Chapter 1, Methods.

Analysis of physiological characters, X-ray irradiation and benlate treatment

These methods are described in Chapter 1 and Chapter 2.

Isolation of purified mtDNA and nuclear DNA

Mitochondrial and nuclear DNA were prepared following the same procedure described in Chapter 2 for the isolation of rDNA. Purified mtDNA was cleaved with *TaqI* in buffer containing 6 mM Tris/HCl pH 7.5, 6 mM MgCl₂, 6 mM mercaptoethanol. Fragments were resolved in 1% agarose gel (see Chapter 2, Methods) and photographed, under illumination by short-wave U.V. lamps (General Electric, USA) using a Polaroid type 665 P/N film and a Polaroid MP4 camera with a Kodak No. 23A orange filter. Conditions for *EcoRI* digestion of DNA have been described in Chapter 2 Methods. Transfer, labelling and hybridisation of DNA were also performed following the procedures given in Chapter 2 Methods with the only modification that, when ^32P labelled mtDNA was used as a
probe, hybridisation was carried out at 55°C.

**Ethidium bromide production of petite colonies**

A drop of ethidium bromide solution (10 mg/ml) was placed on a GYP plate. After the drop had diffused into the agar a loopful of a culture grown overnight at 30°C was streaked across the dye and the plate incubated at 25°C. After 4-7 days incubation, cells from the margin of growth near the dye were streaked away from the ethidium bromide. Small single colonies were picked after a further 4-7 days incubation at 25°C, resuspended in water and dropped on GlyYP and GYP plates to test their respiratory competence.

**RESULTS**

A. **Isolation of prototrophic colonies between S. cerevisiae *Fep*− and K. lactis**

Fusion was induced by means of PEG (30%), in the presence of CaCl$_2$ (75 mM), between protoplasts of *Fep*− (*ade1 arg4-16*) and K25 (*lys ura*). Although formation of protoplasts was rapid and adequate in both strains, addition of PEG to the mixed protoplasts did not promote the impressive agglutination observed during fusion experiments between D6ep− and K25. Only under microscopic examination could many small aggregates be seen. Likewise, cell wall regeneration on MMKA medium
Fig. 3-1. Abnormal cell morphology of fusion products (A), cells of K25; (B) and (C) fusion products at the same magnification.
was very poor for protoplasts of strain Fed− (approx. 0.1%).

Fusion products were selected on MMK plates incubated at 25°C for 3 weeks. After this time, six small colonies had grown on the selective medium. These were tested on MM and GYP, at 25°C. Three isolates were inviable, as no growth appeared on either of the two media, whereas the other three colonies (KF3, KF4 and KF6) showed good growth on both media after 4 days. The three subclones had similar colony morphology on selective medium, that is colonies of different size were present in all isolates. Moreover, KF4 and KF6, but not KF3, appeared to be a mixture of pink and white colonies on MM, where the pink colonies produced a pulcherrimin-like pigment which diffused into the medium. Some KF6 colonies presented pigmentation also on GYP, a medium which does not induce production of pulcherrimin in K25. Examination of cell morphology in all fusion products, under the light microscope, revealed that many cells had unusual shape and larger size than K25 or Fed− (Fig. 3-1). When a loopful of each culture growing on selective plates was resuspended in liquid MM and incubated at 25°C for 3-4 days, only a few cells with abnormal cell morphology were observed. Similarly, colony morphology of these secondary clones on MM solid medium was more regular. The stability of the prototrophic isolates was also analysed. Cells from
stationary cultures in liquid MM, after appropriate
dilution, were spread in equal numbers on MM and GYP
plates. The same number of colonies arose on both types
of medium.

B. Analysis of prototrophic colonies

(1) Physiological characteristics. All the
physiological traits which were examined in fusion
products between K25 and D6ep− (see Section B of Results
in Chapter 2) were also analysed in KF3, KF4 and KF6.
Maltose assimilation and splitting of arbutin were
present in all three protoplasts, although formation of
brown colour in the arbutin medium inoculated with KF3
was slower than for K25, KF4 and KF6 (8-10 days). KF4
and KF6 showed enhanced production of pulcherrim in
respect to K25, whereas KF3 did not exhibit this
property. Growth at 37°C was not tested since KF3 and
KF4 grow poorly at 30°C and KF6 cannot grow at this lower
temperature. None of the fusion products grew under
anaerobic conditions. Finally, in Fig. 3-2 and Fig. 3-3
are presented the results of tests on cycloheximide and
cryptopleurine sensitivities. It can be seen that all
three prototrophic clones resemble K25 for resistance to
100 μg/ml cycloheximide (Fig. 3-2) but, also, behaved
like Fep− in the presence of 0.5 μg/ml cryptopleurine
(Fig. 3-3).

Attempts were made to establish the ploidy of KF3,
Fig. 3-2. Resistance to cycloheximide in fusion products.

(A) NBA control plate; (B) NBA plate containing 100 μg/ml cycloheximide.

(1) KF6; (2) KF4; (3) KF3; (4) KD11; (5) Pep⁻; (6) K25.
Fig. 3-3. Sensitivity to cryptopleurine in fusion products. Each plate contains different concentrations of the drug:
(A) 0.1 μg/ml; (B) 0.2 μg/ml; (C) 0.5 μg/ml; (D) 1.0μg/ml.
(1) K25; (2) Fep−; (3) KD11; (4) KF3; (5) KF4; (6) KF6.
KF4 and KF6, however reliable values of DNA content per cell could not be estimated since counting of samples for DNA estimation was hampered by the clumping of cells always present in cultures of the fusion products and also by their unusual cell morphology (see Fig. 3-1). Studies on sensitivity to ionising radiation and isolation of mitotic segregants are presented below.

(2) Analysis of X-ray sensitivity - The survival curves to X-ray irradiation of the prototrophic clones and the parental strains are presented in Fig. 3-4. Linear response to the radiation was observed in all cases. Furthermore, all fusion products were more sensitive to X-ray irradiation than K25. However, some small colonies were produced by the radiation in all three prototrophs. Segregants could be identified among the small colonies. As shown in Table 3-1, KF3 and KF6 segregated only lys, ura or lys ura phenotypes, while KF4 gave rise also to an ade lys segregant. This appeared as a red sector in a colony which, when subcloned, was composed of cells forming all white or all red colonies. A single white colony and a single red colony from the subclone were analysed. Whereas the white clone was still prototrophic, the red colony now required adenine and lysine for growth. To test if this result could be reproduced, KF4 was submitted again to X-ray irradiation and another segregant with the same phenotype
Fig. 3-4. Dose-response curves to X-ray irradiation.
The curves represent: • K25; □ Fe⁰⁻; △ KF3; X KF4; o KF6.
TABLE 3-1. Mitotic segregation in "small colonies" produced after X-ray exposure of fusion products

<table>
<thead>
<tr>
<th>Fusion products</th>
<th>Total No. of small colonies</th>
<th>Proto-trophic colonies</th>
<th>Auxo-trophic colonies</th>
<th>lys</th>
<th>ura</th>
<th>lys</th>
<th>ade</th>
<th>ura</th>
<th>lys</th>
</tr>
</thead>
<tbody>
<tr>
<td>KF3</td>
<td>7</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KF4</td>
<td>25</td>
<td>21</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>-</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KF6</td>
<td>17</td>
<td>16</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

TABLE 3-2 Mitotic segregation of phenotypes other than ade lys in KF4.2r induced with benlate

<table>
<thead>
<tr>
<th></th>
<th>Benlate 150 µg/ml</th>
<th>Benlate 200 µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prototrophic colonies</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Auxotrophic colonies</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>ade</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>lys ura</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>ade lys ura</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>
was obtained. Since this phenotype was segregated amongst a few hundred survivors and, also, in both cases two markers were simultaneously produced, it could be discounted that the ade lys requirements had arisen from mutational events induced by the radiation. The two isolates were called KF4.1r and KF4.2r and used in further studies.

(3) Characterisation of KF4.1r and KF4.2r - The appearance of a red ade phenotype simultaneously to the requirement for lysine in segregants of KF4 might have resulted from suppression of part of the K. lactis genetic information present in KF4 (suppression or 'switch off' of S. cerevisiae Lys+ gene and 'switch off' of K. lactis Ade+ gene). On this assumption other properties of KF4.1r and KF4.2r were analysed to verify if the two segregants underwent any other phenotypical changes in respect to KF4. However, all the physiological traits characteristic of KF4, namely maltose assimilation, resistance to cycloheximide, absence of growth at 37°C and splitting of arbutin, were unchanged in KF4.1r and KF4.2r.

Subsequently, the origin of the ade lys segregants was investigated by studying the effects of the haploidising agent benlate on KF4.2r. Small and sectored colonies generated by plating KF4.2r on GYP containing 150 μg/ml or 200 μg/ml benlate were analysed
and the results are presented in Table 3-2. Two white colonies which did not require adenine could be isolated, thus indicating that a complex condition is present in KF4.2r.

(4) Analysis of specific DNA sequences in prototrophs by molecular probing

In order to gain information on the genetic constitution of the prototrophic colonies, a molecular approach similar to that applied for analysis of the fusion products between K25 and D6ep⁻ was undertaken. The patterns of hybridisation of BamHI digests of whole cell DNA from K25, Fep⁻ and from the prototrophic isolates to ³²P labelled rDNA are shown in Fig. 3-5. It can be seen that only the K. lactis ribosomal cistrons are present in all fusion products. Similarly, hybridisation of EcoRI cleaved total DNA from the same samples to ³²P labelled YEp6, X-4 or pYel61A3 revealed that neither the his3 sequence, nor the 2 µm DNA or Tyl-161 elements from the Fep⁻ strain could be found in any of the three prototrophs. Unfortunately, a plasmid containing the ade1 sequence of S. cerevisiae was not available. The use of such a sequence as a probe could be very informative on the origin of the red ade phenotype recovered after X-ray irradiation of KF4.
Fig. 3-5. Hybridization patterns of *BamHI* digested total DNA from fusion products to $^{32}$P-labelled rDNA.

(a) K25; (b) KF3; (c) KF4; (d) KF6; (e) FeP$^-$.
C. **Induction of the petite mutation in prototrophic clones and their segregants**

As KF3, KF4 and KF6 were obtained from fusion between the petite-negative *K. lactis* and a *ρ°* strain of *S. cerevisiae*, these isolates should contain only *K. lactis* mtDNA. In fact, cleavage of purified mtDNA from K25 and the fusion products with the restriction nuclease *TaqI* gave fragments in the same number and size for all samples (Fig. 3-6). Attempts to induce petite colonies by ethidium bromide treatment in KF3, KF4 and KF6 were unsuccessful. All small colonies isolated after exposure of the prototrophs to E.B., at a concentration (10 mg/ml) which produces petites at very high frequency in petite-positive yeasts, had not lost their ability to grow on GlyYP. By contrast, when the same treatment was applied to KF4.1r and KF4.2r, colonies incapable of growth on a non-fermentable carbon source were found.

To test if the respirator-deficient clones were cytoplasmic petites produced by large deletions or complete loss of the mtDNA, *EcoRI* cleaved whole cell DNA from these colonies, K25 and KF4.2r, as well as purified mtDNA from K25 as a control, was hybridised to $^{32}\text{P}$-labelled mtDNA from K25. It can be seen in Fig. 3-7 that all four respirator-deficient isolates lack all fragments present in mtDNA of *K. lactis* (lane a), that is they are all petites of the *ρ°* type. However, an interesting finding could be deduced from Fig. 3-7: a
Fig. 3-6. Patterns of mtDNAs from K25 and fusion products after digestion with TaqI. Fragments separated on a 1% agarose gel.

(a) K25; (b) KF3; (c) KF4; (d) KF6; (m) pBR322 marker digest.
Fig. 3-7. Hybridisation patterns of whole cell DNA from E.B.-induced petites of KF4.1r and KF4.2r to $^{32}$P labelled K. lactis mtDNA. All samples except (h) were cleaved with EcoRI. In (a) purified K. lactis mtDNA was used as a control.

(a) K25 mtDNA; (b) K25; (c) KF4.1r5; (d) KF4.2r1;
(e) KF4.2r5; (f) KF4.2r7; (g) and (h) KF4.2r.
Fig. 3-8. Hybridisation patterns of *EcoRI* digested purified nuclear (a) and mitochondrial (b) DNA from *K. lactis* to $^{32}$P labelled mtDNA containing the *oxi1* (A) or *oli1* (B) sequences of *S. cerevisiae*. 
fragment of about 5.5 kb in size (Wesolowski et al., 1981) hybridised to $^{32}$P-labelled K. lactis mtDNA in all samples, except for the sample of purified K25 mtDNA. It should also be noted that undigested whole cell DNA from KF4.2r (lane h) did not show hybridisation to a discrete fragment. Considering all these data together, it can be concluded that a sequence presenting high homology to part(s) of the mtDNA is present in the nuclear DNA of K. lactis. This was confirmed by analysis of hybridisation patterns of purified nuclear DNA and mtDNA, digested with EcoRI, to $^{32}$P-labelled mtDNA from S. cerevisiae petites retaining only a particular sequence of mtDNA. Hybridisation of EcoRI nuclear DNA and mtDNA from K25 to labelled mtDNA from strain DS302 of S. cerevisiae containing only the sequence for oxil (gene for cytochrome oxidase subunit II) showed that a fragment of the nuclear DNA presented good homology to the oxil sequence and also had a different mobility from the K. lactis mitochondrial fragment containing oxil (Fig. 3-8A). When the same digests were hybridised to a probe of oxi2 or oli1, fragments of the mtDNA showed hybridisation whereas there was no homology between the two probes and the nuclear DNA (Fig 3-8B).

D. Spontaneous petite formation in KF4.1r and KF4.2r

Spontaneous petites from KF4.1r and KF4.2r were sought by plating cultures of the two clones on GGYP. On
Fig. 3-9. Colony morphology of KF4.1r on GGYP medium. (A) magnification of part of (B).
this medium, which contains a limiting amount of a fermentable carbon source, petite mutants can grow only for a small number of generations, thus forming small colonies. Approximately $10^4$ colonies for each of the two isolates were screened but no petites could be isolated. Moreover, an unusual feature was observed in colonies of both strains after two weeks incubation at 25°C. Most colonies presented white papillae and white or, more frequently, pink sectors (Fig. 3-9). This was never observed when KF4.1r and KF4.2r were grown on GYP medium. A few white papillae were subcloned and single white or red colonies from each subclone were tested for their requirements. While the red subclones were still requiring adenine for growth, the white ones were now only lys. Furthermore a large white sector from a colony on the original GGYP medium was found to be prototrophic.

**DISCUSSION**

Unlike the *S. cerevisiae* strain D6ep<sup>-</sup>, the Fep<sup>-</sup> mutant of the same species produced stable prototrophs upon fusion with *K. lactis*. As fusion between *S. cerevisiae* and *K. lactis* has never been reported previously, insufficient data are available to date which could elucidate why this discrepancy was found.
Characterisation of all the prototrophs revealed that these were mostly *K. lactis*-like, as already observed for the *lys ura arg* fusion products between K25 and D6ep<sup>-</sup>. Therefore, a "dominance" of the *K. lactis* genome appears to be a common feature of interactions between the *S. cerevisiae* and *K. lactis* genomes. This observation cannot be understood at present. Expression of resistance to cryptopleurine at the same level as for *S. cerevisiae* in the prototrophic colonies was the only exception to this dominant behaviour of *K. lactis.*

Genetic analysis of KF3, KF4 and KF6 by means of X-ray irradiation showed that segregation of the *lys, ura* or *lys ura* markers could be obtained, although KF4 and KF6 segregated fewer auxotrophic phenotypes than KF3. Moreover, KF4 produced a red *ade lys* segregant amongst a few hundred cells which survived exposure to the radiation. However, no arginine requiring colonies were found. These data, together with the observed absence of ribosomal DNA, *his3* or Tyl-161 element sequences from *S. cerevisiae*, can be interpreted by postulating that only portions of the *S. cerevisiae* genetic information are retained in the fusion products. Unlike the *lys ura arg* fusion products between K25 and D6ep<sup>-</sup>, however, KF3, KF4 and KF6 must contain at least enough *S. cerevisiae* information to complement the *lys ura* markers of K25.

In regard to the *ade lys* segregants from KF4 it is not clear why both colonies, although independently
isolated, had the same phenotype. The recovery of ade or lys ura clones from benlate treatment of KF4.2r, as well as the segregation of lys from irradiated KF4, indicates that there is no close linkage between the ade and the lys markers in KF4. Furthermore, since benlate treatment of KF4.2r produced colonies which no longer required adenine, it is probably incorrect to define KF4.1r and KF4.2r as "segregants" of KF4.

Comparable results were found in B. subtilis. Although a more complex genetic system was herein studied and no conclusive experimental evidence could be provided on this problem, there is indication from the results presented that the loss of gene expression, but not of genetic material, encountered in fusion products between some bacterial species (Hotchkiss and Gabor, 1980) could also be responsible for the isolation of ade lys phenotypes from KF4. In other words, KF4.1r and KF4.2r did not loose K. lactis as well as S. cerevisiae genetic information to reveal the ade and lys markers but loss of expression of the wild-type alleles of these two genes may have been produced by the radiation or by benlate. It is not understood, however, how exposure to X-ray irradiation or to benlate could result in such an unusual phenomenon.

Support to this hypothesis was also provided by the finding that, although KF4 could not be induced to form petite mutants, KF4.1r and KF4.2r behaved like petite-
positive strains in the presence of ethidium bromide, that is $\rho^0$ petites were isolated from both clones. Furthermore, when spontaneous petites were sought from KF4.1r and KF4.2r by plating their untreated cultures on a medium which selects for $\rho^-$ mutants, white papillae were formed which had lost the requirement for adenine and, in some cases, were now prototrophic.

It can be concluded that expression of the K. lactis genome in KF3, KF4 and KF6 is "dominant", especially when these isolates or their "segregants" are under some selective pressure. Secondly, these fusion products are not diploid, but contain at least a few genes from Fep$, as indicated by the complementation of the requirements present in K25, the decreased sensitivity to cryptopleurine and also the ability to produce $\rho^0$ petites.

In this regard, it must be stressed that the mtDNA of a petite-negative yeast (K. lactis) can be completely deleted without affecting cell viability, provided that nuclear information from a petite-positive yeast (S. cerevisiae) is also present in the cell. This information could allow increased fermentation.

It was also discovered that a sequence of at least one mitochondrial gene (oxiI) is also present in DNA of nuclear buoyant density. Since whole cell undigested DNA did not show the presence of episomal DNA, it can be assumed that the common sequence is in the nuclear
DNA. This could be similar to what has been reported for the gene for ATPase subunit 9 in *Neurospora crassa*. In fact, in this organism the gene corresponding to the mitochondrial *oli1* locus of *S. cerevisiae* is in the nucleus but a homologous sequence interrupted by a stop codon is also present in its mtDNA (Fox, 1981).

Finally, spontaneous petites could not be isolated from KS4.1r or KS4.2r by screening $10^4$ colonies on selective medium. It is evident that the process of spontaneous petite formation in these isolates does not occur at a high frequency, as observed in *S. cerevisiae*. Since the mechanism of petite production appears to be the same in both spontaneous and induced mutants in *S. cerevisiae* (Heude et al., 1979), more information is needed on the complex genetic constitution of the fusion products before this aspect can be clarified.
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APPENDIX
<table>
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<tr>
<th>Species</th>
<th>Strain</th>
<th>Tissue Genotype</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kluveromyces lactis</td>
<td>25</td>
<td>a lys, ura</td>
<td>U.V. induced mutant from segregant of cross W599C x W231B (A. Herman, Northern Region Research Laboratories, Peonia, U.S.A.).</td>
</tr>
<tr>
<td>WM52</td>
<td>α</td>
<td>ade his</td>
<td>A. Herman, Northern Region Research Laboratories, Peonia, U.S.A.</td>
</tr>
<tr>
<td>Pachytespora transvaalensis</td>
<td>CBS 2186</td>
<td>prototroph</td>
<td>Centraal Bureau voor Schimmelcultures.</td>
</tr>
<tr>
<td>Saccharomyces cerevisiae</td>
<td>T</td>
<td>a ade8-18 lys2</td>
<td>F. Fogel, Berkeley, U.S.A.</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>a ade1 arg4-16</td>
<td>&quot;                       &quot;</td>
</tr>
<tr>
<td></td>
<td>500ep</td>
<td>a ade1 arg4-16</td>
<td>E.B. induced petite from a segregant of FXT.</td>
</tr>
<tr>
<td></td>
<td>D6</td>
<td>α arg8 met</td>
<td>D. Wilkie, University College, London.</td>
</tr>
<tr>
<td></td>
<td>X1049-9c</td>
<td>a arg8 trp1 ura3 his8 asp6</td>
<td>Yeast Genetic Stock Center, Berkeley, U.S.A.</td>
</tr>
<tr>
<td></td>
<td>JB82</td>
<td>α arg8 ade2-2 gal2</td>
<td>&quot;                       &quot;</td>
</tr>
<tr>
<td>Species</td>
<td>Strain</td>
<td>Genotype</td>
<td>Origin</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>-----------</td>
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<tr>
<td><em>Saccharomyces cerevisiae</em></td>
<td>DS302</td>
<td>oxi1</td>
<td>A. Tzagoloff, Columbia University, New York.</td>
</tr>
<tr>
<td></td>
<td>DS31</td>
<td>oxi2</td>
<td>&quot;</td>
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<tr>
<td></td>
<td>DS400/A3</td>
<td>oli1</td>
<td>&quot;</td>
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<tr>
<td><em>Saccharomyces exigus</em></td>
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<td>prototroph</td>
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<td><em>Saccharomyces telluris</em></td>
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<td><em>Saccharomyces unisporus</em></td>
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<td><em>Torulaspora globosa</em></td>
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<tr>
<td><em>Zygosaccharomyces mrakii</em></td>
<td>CBS4218</td>
<td>&quot;</td>
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</tr>
</tbody>
</table>
A. STRAINS

The strains used in this study, their genotypes and sources of origin are listed in Table A-1.

B. MEDIA

i. MM medium contains per litre: 6.7g yeast nitrogen base (Difco) and 20g glucose.

ii. YP media contain per litre: 10g bactopeptone (Difco), 5g yeast extract (Difco) and 3g KH$_2$PO$_4$. For the following media YP was supplemented with (/L): GYP, 20g glucose; GlyYP, 40 ml glycerol; GGYP, 40 ml glycerol and 2g glucose.

iii. NBA medium was prepared by dissolving 0.67g yeast nitrogen base (Difco) in 90 ml distilled water. After addition of 1.5g purified agar (Noble agar, Difco) the medium was autoclaved and cooled to approx. 40°C. 10 ml of a 10% solution of the carbon compound sterilised by passage through a Millipore filter were then added to the medium, mixed and poured into petri dishes.

iv. Indicator medium contains per litre: 1.5g bactopeptone, 1.5g yeast extract, 3g KH$_2$PO$_4$, 20g glucose and 30 mg Gurr's methylene blue.

v. Malt agar medium contains per litre: 25g malt extract and 15g agar.
C. CHEMICALS

Agarose Type II (No. A-6877) from Sigma, PO Box 14508, St. Louis, MO 63178, U.S.A.

Arbutin (No. A-4256) from Sigma, ibid.

Caesium chloride (sequanal grade) from Pierce Chemical Co., Rockford, Il. U.S.A.

Chloramphenicol from Sigma, ibid.

Cryptopleurine from Chemsea Manufacturing Pty. Ltd., Sydney, Australia.

Cycloheximide from Calbiochem, San Diego, CA., 92112, U.S.A.

DNA polymerase I (Klenow fragment) from Boehringer, Mannheim, W. Germany.

Ergosterol from BDH Chemicals Ltd., Poole, England.

Ethidium bromide (2,7-diamino-10-ethyl-phenyl-phenanthridium bromide) from Calbiochem., San Diego, CA., 92112, U.S.A.

Hoechst compound (H33258) - Bisbenzimide H33258 from Riedel De Hēen AG Seelze-Hanover.

Iso-amyl alcohol (UNIVAR) AR from Ajax Chemicals, Sydney, Australia.

Lysozyme from Calbiochem-Behring Corp., La Jolla, CA., 92037, U.S.A.

Propan-2-ol (ANALAR) AR BDH Port Fairy, Vic., Australia.

vi. 1 L broth contains per litre: 10g bactotrypeptone (Difco), 5g yeast extract and 5g NaCl.

Media were solidified by the addition of 15g/litre agar (Difco).
RNAaseA from Worthington Biochemical Corp., Freehold, New Jersey, 07728, U.S.A.

Sarkosyl-NL-30 from CIBA Geigy, St. Leonards, N.S.W., Australia.

Tween 80 from Lab. Supply Ptd. Ltd., Sydney, Australia.

Zymolyase (60,000) from Seikagaku Kogyo Co., Ltd., Japan.

All other reagents used were AR grade.

Restriction nucleases were locally produced according to the method of Greene et al. (1978). Plasmid X-4 was prepared by Dr. G. D. Clark-Walker. Plasmid YEp6 was a gift from Dr. R. W. Davis, Stanford University, Berkeley and plasmid pYel61A3 was a gift from Dr. J. Carbon, University of California, Santa Barbara.