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CHARACTERISATION OF MALATE-DEPENDENT MUTANTS OF THE YEAST *SCHIZOSACCHAROMYCES MALIDEVORANS*

Department of Microbiology and Genetics
Massey University

A thesis presented in partial fulfilment of the requirements for the degree of Masters in Science in Genetics at Massey University

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ABSTRACT

The phenotypes of several UV induced malate-dependent mutants of *Schizosaccharomyces malidevorans* were characterised and compared to the previously characterised malate-dependent strain, *S. malidevorans* #11. This strain uses less glucose than the wild type, has an extended life in liquid media, does not sporulate readily and turns indicator medium blue.

The malate-dependent mutant strains were analysed for their fermentation characteristics, their complementation groups and their chromosomal patterns on a transverse alternating field electrophoresis apparatus (TAFE). The fermentation patterns of all of the strains were similar to #11. There appear to be three complementation groups involved in the malate-dependent phenotype.

The TAFE patterns and subsequent Southern Hybridisation showed that the malate-dependent mutants had a decreased mobility of chromosome II, while chromosomes I and III were not altered. The chromosome II alteration varied between the different malate-dependent mutants but fell into a small number of discrete patterns.

LIST OF FIGURES

LIST OF TABLES

LIST OF PLATES

ABBREVIATIONS

Abbreviations are as described in the text. Those not described are "accepted" abbreviations (*Biochem. J.* 1983 **209** :1-27).

LIST OF CONTENTS

CONTENTS PAGE

CONTENTS PAGE

1. INTRODUCTION

1.1. *SCHIZOSACCHAROMYCES*

1.1.1. The Genus

Schizosaccharomyces was first isolated by Lindner in 1893 from an African beer made from millet and four species have subsequently been described, *S. japonicus, S. octosporus, S. malidevorans,* and *S. pombe.* They are distinguished on the basis of morphology, such as the number of spores in an ascus (four to eight), and their differing abilities to use the sugars maltose, melibinose and raffinose (Slooff, 1970).

Schizosaccharomyces are ascosporogenous yeasts which, uniquely among such yeasts, reproduce vegetatively by binary fission rather than by budding. They have a predominantly haploid life cycle with conjugation being immediately followed by meiosis and the haploid spores are formed within an ascus (Slooff, 1970). Zygotes can occasionally germinate into diploid colonies but these are unstable (Leupold, 1970).

Sipiczki *et al* (1982) proposed that *S. malidevorans* is a variety of *S. pombe* because the two species are interfertile and Bridge and May (1984) suggested that there was insufficient distinction based on numerical classification between the two to justify them as different species.

1.1.2. *Schizosaccharomyces pombe*

S. pombe is the type species for the *Schizosaccharomyces* genus. Cells are either homothallic or heterothallic with homothallic strains being self-fertile. Heterothallic strains can mate with homothallics or strains of opposite mating type. After conjugation zygotes sporulate to give four ascospores (Gutz *et al*, 1974).

1.1.3. *Schizosaccharomyces malidevorans*

S. malidevorans was first isolated from Listan grapes grown in Spain in 1964. It is a homothallic yeast which produces four ascospores. While closely related to *S. pombe,* it differs in both its inability to ferment maltose and in the rough outer membrane on its spores (Rankine and Fornachon, 1964). It is a high malate-metabolising yeast, degrading close to 100% of L-malate (Rodriguez and Thornton,1988).

1.2. WINE MAKING

Wine making involves a series of processes: harvesting and crushing of grapes, fermentation reactions, and finally, bottling. The fermentation process is crucial to the production of a good wine. Two fermentation processes have been used for many years; one is alcoholic and the other involves malolactic fermentation (MLF) (Marrison, 1962).

1.2.1. Biological Deacidification

For dry reds and some white wines alcoholic fermentation is followed by MLF. MLF is a bacterial secondary fermentation. The reaction is catalysed by NAD dependent malate carboxylase from lactic acid bacteria which occur in the natural microbial flora of the grapes. *Leuconostoc oenos* is the commercially used lactic acid bacterium.

MLF is especially important in colder environments where acids (mostly malic and tataric) are not completely metabolised during ripening. It also stabilises the wine and adds flavour. Deacidification, and in consequence reduction in sour taste, occurs by conversion of L-malic acid (a dicarboxylic acid) to lactic acid (a monocarboxylic acid) and CO_2 (Kunkee, 1974). The presence of L-malic acid in wine also allows the growth of bacteria after bottling which leads to sediment formation, cloudiness and fizziness, so deacidification also confers microbial stability (Williams et al, 1984). Other end products of the MLF bacteria eg acetaldehyde and acetic acid, add to the wines flavour and sensory quality (Kunkee, 1974).

MLF however, is time consuming and expensive. It takes 100 to 200 days depending on whether natural or commercial induction is used and it is difficult to control. Spoilage due to growth of other bacteria or yeasts can also occur.

1.2.2. Chemical Deacidification

Chemical methods are an alternative means to deacidify wine. Common examples are: amelioration (the addition of sugar and water to the juice); neutralisation by the addition of calcium carbonate; and double salt deacidification where tartrate and malate are coprecipitated as a calcium double salt. The disadvantages though are that the wine is considered a falsification, there is a salty taste due to large quantities of calcium malate remaining in solution, and the wine needs to be held for a long time before bottling (Beelman and Gallander, 1979).

1.2.3. *Schizosaccharomyces* in Wine Making

Schizosaccharomyces convert malate to ethanol and CO_2 anaerobically. Being strong alcohol fermenters they resemble wine yeast, but they have a higher optimum growth temperature and are more SO_2 resistant. Alcoholic production appears to be greater in *Schizosaccharomyces* wines but high ethanol levels delay malic acid degradation (Beelman and Gallander, 1979).

Wines produced using *Schizosaccharomyces* have an inferior flavour, *S. malidevorans* does so due to the evolution of H_2S. *Schizosaccharomyces* is also a slower fermenter and grower and so is overgrown by *Saccharomyces* in mixed cultures.

Consequently, researchers have attempted to construct high malate-metabolising wine yeast strains to use in alcoholic fermentation. Williams *et al* (1984), cloned and transferred the gene for malolactic activity from *Lactobacillus delbrueckii* to a strain of *Saccharomyces cerevisiae*. Differences in the expression systems of procaryotes and eucaryotes however resulted in low expression. This could be overcome if the malate utilising genes from *S. malidevorans* can be isolated and cloned into wine yeasts. Such an approach requires further characterisation of the system and the number of genes involved in the malate utilising phenotype needs to be determined.

1.2.4. *Schizosaccharomyces malidevorans* #11

The malate-dependent (mig^-) mutant #11 was isolated by ultraviolet (UV) light mutagenesis of wild type 442 and plating onto an indicator (IND) medium. The mutant #11 colonies appeared light blue whereas the wild type colonies were green (Rodriguez and Thornton, 1988).

This mutant #11 has several improved features for wine making. It uses malate at approximately twice the rate of wild type 442. This is coupled with a decreased utilisation of the sugar carbon source and it does not produce the off-flavours due to H_2S production (Rodriguez and Thornton, 1989). #11 may be suitable for an initial fermentation to remove malate before inoculation with a wine yeast.

1.2.5. The #11 Phenotype

The phenotype of #11 has been characterised (Rodriguez and Thornton, 1988; Cambourn-Theewis, 1990). #11 is malate-dependent but requires a carbon source such as glucose, it uses 100% of L-malate and does not sporulate readily. #11 cannot use malate above a certain level (possibly suggesting that it cannot control malate transport) (Cambourn-Theewis, personal communication). It has a slower growth rate than the wild type but an extended stationary phase in liquid medium, probably due to

the production of fewer inhibitory products from a lower glucose utilisation. It also has a higher pH maximum and is homothallic.

Four other mig$^-$ mutants have been isolated by UV mutagenesis (Lubbers, 1987). These mutants also require malate for growth, are homothallic, sporulate poorly and have a blue colony colour on IND medium but they have not been characterised to the extent that #11 has. Genetical analysis and fermentation studies need to be done on these four mutants to determine whether the #11 phenotype is characteristic of all the mig$^-$ mutants.

1.3. MALATE TRANSPORT

Transport of malate is likely to be important in malate utilisation, but little is known about it in *S. pombe* or *S. malidevorans*. Other yeasts have been studied and their systems characterised. These yeasts may be provide some insight into transport in the *Schizosaccharomyces* yeasts.

Baranowski and Radler (1984) studied malate transport in *Zygosaccharomyces bailii*. This species can completely degrade malate anaerobically in the presence of glucose, but only partially in grape juice. Malate is transported in *Z. bailii* by an inducible system of facilitated diffusion. Like the carrier system for glucose, the malate carrier protein is inducible by glucose and does not work against a concentration gradient. L-malate is rapidly and specifically transported, entering the cells four times faster than D-malate. This stereospecificity of malate transport in the cells of *Z. bailii* supports the carrier protein model.

Baranowski and Radler also discovered that the malate transport system in *Sacc. cerevisiae* occurs by passive diffusion. Both enantiomers of malate enter *Sacc. cerevisiae* cells in equal amounts and no substrate saturation was observed (unlike in the cells of *Z. bailii*).

Neurospora crassa and *Kluyveromyces lactis* have a single active, inducible transport system for L-malate, fumarate and succinate, which is subjected to catabolite repression (Osothsilp and Subden, 1986 b).

Using malate transport-deficient mutants Osothsilp and Subden, (1986 b) deduced that malate transport in *S. pombe* is active, constitutive and mediated by a carrier protein. The carrier was shown to be energy dependent as it can be inhibited by inhibitors of electron transport. The energy probably comes from the carbon source, glucose, and transport is pH and temperature dependent.

Glucose transport in *S. malidevorans* wild type and #11 is similar but L-malate transport in the mutant was derepressed so malate was transported into the cells at a

faster rate. This derepression was seen in the absence and presence of glucose (Cambourn-Theewis 1990).

1.4. MALATE METABOLISM

The ability of yeasts to metabolise malic acid is important in the wine industry but the mechanisms of malate metabolism are not completely understood, especially in *S. pombe* and *S. malidevorans*. The mechanisms in other yeasts may provide an insight into malate metabolism in the *Schizosaccharomyces* yeasts. Malate metabolism predominantly involves malic enzyme (ME).

1.4.1. *Saccharomyces cerevisiae*

Sacc. cerevisiae oxidises L-malate to pyruvate by ME under anaerobic conditions. Pyruvate is then oxidised to acetaldehyde and CO_2 (by pyruvate decarboxylase) and finally ethanol (by alcohol dehydrogenase). Only 16-33% of malate is oxidatively decarboxylated due to low levels of intracellular malate, which enters by diffusion, and the low substrate affinity of ME.

The ME of *Sacc. cerevisiae* is bifunctional, reacting with both malate and oxaloacetate. Oxaloacetate is decarboxylated in the absence of NAD but ME activity requires the presence of both NAD and Mn^{2+} (Kuczynski and Radler, 1982).

1.4.2. *Saccharomyces bailii*

Sacc. bailii is an osmotolerant spoilage organism of juice concentrates which can completely metabolise malate. Malate enters the cell via an inducible carrier protein.

The ME of *Sacc. bailii* is similar to that of *Sacc. cerevisiae* in being non-inducible, having a neutral pH optimum and favouring NAD over NADH as the coenzyme. It differs however, in not requiring Mn^{2+}, in having oxaloacetate decarboxylated by malate dehydrogenase rather than ME and in possessing a five times greater substrate and coenzyme affinity. This increased substrate affinity coupled, with a high intracellular malate concentration, promotes the complete metabolism of malate (Kuczynski and Radler, 1982).

Fumarase was shown to compete with ME for L-malate in *Sacc. bailli*, dehydrating malate to form succinate. Under anaerobic conditions fumarase is inhibited by phosphate so only a little of the malate is consumed by this pathway (Kuczynski and Radler, 1982).

1.4.3. *Schizosaccharomyces pombe*

S. pombe can oxidise a number of intermediates from the Tricarboxylic acid cycle (TCA). Mono- and dicarboxylic acids are oxidised at different rates, while citrate, tartrate and lactate are not oxidised at all (Mayer and Temperli, 1963).

ME in *S. pombe* is specific for L-malate, requiring NAD, Mn^{2+} and a carbon source such as glucose (Mayer and Temperli, 1963). Osothsilp and Subden (1986 a) demonstrated that ME activity occurs both aerobically and anaerobically in *S. pombe*. As in *Aspergillus*, the ME of *S. pombe* is the product of a single gene (Osothsilp and Subden, 1986 a).

1.4.4. *Schizosaccharomyces malidevorans* #11

The ME of *S. malidevorans* is assumed to be similar to *S. pombe* as they are closely related species. Cambourn-Theewis (1990) reported that mutant #11 had a ten fold increase in ME activity over that of the wild type, partly due to the fact that malate enters the cells of #11 faster than in 442.

It is unknown, however, if #11 is defective in malate metabolism and also whether the different malate-dependent mutants are defective in the same gene(s).

1.5. GENETICS OF THE YEAST

S. pombe, being a lab yeast, is genetically well characterised strains, while little is known about the genetics of "wild" *S. malidevorans*. *S. pombe* has three chromosomes and a gene map has been constructed (Gygax and Thuriaux, 1984). Several auxotrophic marker strains are also available. *S. malidevorans* was isolated in 1964 and has been less characterised. Fewer auxotrophic marker strains are available and there is no gene map.

1.5.1. The Mating Types of *Schizosaccharomyces*

S. pombe strains are either homothallic or heterothallic. Homothallic strains (h^{90} cells) contain a mixture a h^+ and h^- cells and vegetatively growing cells switch their mating type once every few cell divisions. Heterothallic strains exist as either of the h^+ or h^- mating types. They are stable, not changing their mating type, although h^+ cells can change to h^{90} cells at a reasonable frequency. Mating requires both mating types and so homothallic strains are self-fertile (Gutz *et al*, 1974).

S. malidevorans strains are homothallic. The #11 mutant also appears to be homothallic as it mates with both h^+ and h^- strains.

1.5.2. The Genetics of *S. malidevorans* #11

S. pombe can complement the malate-dependence of #11, suggesting that malate-dependence is a recessive mutation (Lubbers, 1987) although the ploidy of the mutant is unknown. Crosses with *S. pombe* (and also *S. malidevorans*) give a low frequency of mating, very low spore viability and aberrant segregation ratios (Cambourn-Theewis, 1990). In these crosses the malate-dependence and mating-type homothallism seem to segregate together. At present no heterothallic, malate dependent cells have been isolated, although attempts have been made to mutagenise heterothallic strains (Lubbers, 1987).

The complementation groups of #11 and some other mig$^-$ mutants have been studied. Parker (1988) and Rodriguez (pers. comm.) suggested the presence of at least two complementation groups for malate-dependence, implying that there are at least two genes involved in the malate-dependent phenotype. Their results were inconclusive however (see discussion). Further characterisation of possible complementation groups needs to be carried out, but this requires the presence of more auxotrophic markers in the strains.

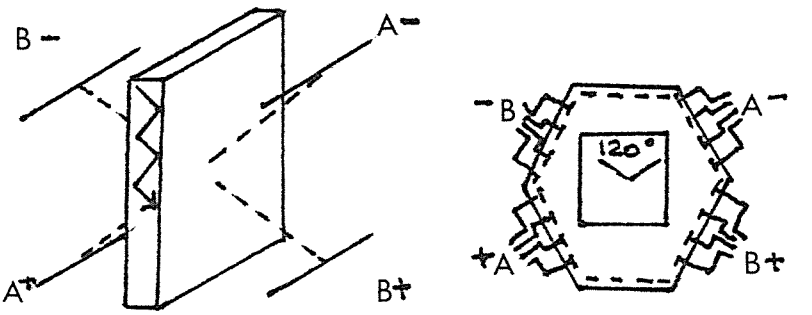
1.6. GEL ELECTROPHORESIS

Conventional gel electrophoresis is used to separate DNA molecules by size. The DNA moves through an agarose gel in response to an electric field towards the anode (positive electrode). Smaller molecules move faster than bigger molecules creating a banding pattern in the gel. DNA molecules larger than 50 kb cannot be resolved however, but the use of two alternating electric fields can separate very large molecules (Schwartz *et al* 1982). This is called pulsed field gel electrophoresis (PFGE). Many variations of PFGE systems have been developed (Lai *et al*, 1989) and two are used in this study; TAFE (transverse alternating field electrophoresis) and CHEF (contour clamped homogeneous electric field).

1.6.1. TAFE and CHEF Electrophoresis

TAFE is different to most PFGE in that the gels are run vertically submerged in a buffer chamber. TAFE uses 2 homogeneous fields separated at an initial angle of 115°. The electric fields are orthogonal to the gel causing the DNA to zig-zag through the thickness of the gel rather than over the plane of the gel (Beckman; Fig 1 a). TAFE can separate 13 of the 17 chromosomes of *Sacc. cerevisiae*. DNA mobility is not constant because the angle between the electrodes varies from the top (115°) to the bottom (165°) of the gel (Lai *et al* , 1989).

A more recent development is the CHEF system (Bio-Rad) which is able to separate 16 of the *Sacc. cerevisiae* chromosomes. CHEF has 24 electrodes arranged in a hexagonal pattern, with a reorientation angle of 120° so that the DNA migrates in a zig-zag pattern across the face of the gel (Fig 1 b). The electric field is uniform at all parts of the gel so the lanes run straight.



a) TAFE b) CHEF

Fig. 1 Schematic diagram of TAFE and CHEF.

1.6.2. Applications

Chromosomes of various organisms have been separated using PFGE techniques, including, *Sacc. cerevisiae* (Schwartz and Cantor, 1984), the filamentous fungus *Acremonium lolii* (Murray, pers. comm.), and even portions of the human genome (Fulton *et al* , 1989). *S. pombe* chromosomes have been examined (Smith *et al*, 1987), with Fan *et al* (1988) using the rare cutting restriction enzyme *Not* I to construct a map of the *S. pombe* genome.

Chromosomal patterns looked at on TAFE showed that *S. malidevorans* 442 had the same chromosomal pattern as wild type *S. pombe*. The malate-dependent mutant #11, however had a slightly different pattern, chromosome II having shifted position. This could be explained by a DNA duplication or chromosomal translocation (Murray, 1989).

S. malidevorans contains a similar amount of DNA to a standard haploid strain of *S. pombe* implying that it is haploid (Murray, 1989). The mutant #11, however, contained approximately 1.4 times as much DNA as the wild type suggesting it is aneuploid. If it was diploid, the DNA ratio would be expected to be 2. UV survival curves agree with these findings. Wild type 442 gave a similar curve to the haploid *S. pombe* whereas the #11 curve implied that #11 has more DNA than a haploid strain (Murray 1989).

The nature of the chromosomal change, whether it is found in all mig$^-$ mutants and whether it is the cause of the malate-dependence, has not been determined. TAFE offers a way to study the difference between the mig$^-$ mutants of *S. malidevorans* and

the wild type and in combination with restriction digest analysis could provide information on the nature of the change.

1.7. OBJECTIVES

The aims of this project are to investigate both biochemical and genetical features of the mig$^-$ mutants. Specifically this thesis looks at:

1. **Fermentation Patterns**
   (i) enzyme analysis of glucose and L-malate levels
2. **Complementation and Spore Clone Analysis**
   (i) generation of other mig$^-$ mutants by UV mutagenesis
   (ii) introduction of auxotrophic markers into the mig$^-$ mutants
   (iii) identification of complementation groups
   (iv) spore clone analysis of diploids
3. **Chromosome Characterisation**
   (i) characterisation of the mig$^-$ and mig$^+$ mutants on TAFE
   (ii) identification of the chromosome bands using chromosome specific gene probes
   (iii) *Not* I analysis

The identification and characterisation of distinct classes of mig$^-$ mutants will be useful in the study of the molecular basis of the mutation.

2. MATERIALS AND METHODS

2.1. STRAIN LIST

Strain	Genotype	Comment	Original source
Schizosaccharomyces pombe			
972	h^-	wild type	P. Thuriaux CIF-SUR-YUETTE
Spk 1	h^+ ade 6-704		P. Thuriaux
Spk 2	h^- ade 6-704		P. Thuriaux
Spk 3	h^- ura 4		F. Lacroute, CRNS, Strasbourg
131	h^+ leu 1-32		B. Hall, Uni Washington, Seattle
Schizosaccharomyces malidevorans			
442	h^{90} mig^+ [1]	wild type	B. Rakine A.W.R.I.
Spz A	h^{90} mig^+ arg^-	442 mutant	C. Harrod Massey University
Spz L	h^{90} mig^+ lys^-	442 mutant	C. Harrod
#11	mig^- [1]		S. Rodgriguez, DSIR, Palmerston North
XL1A [2]	mig^- ade^-	#11 spore clone	M. Lubbers Massey University
3/dLB	mig^-		M. Lubbers
N1A [3]	mig^- arg^-	3/dLB spore clone	N. Hansen Massey University
N1L [3]	mig^- lys^-	3/dLB spore clone	N. Hansen
THX1U [2]	mig^- ura^-	3/dLB spore clone	N. Hansen
THX1L [2]	mig^- leu^-	3/dLB spore clone	N. Hansen
8/dLB	mig^-		M. Lubbers
N4A [3]	mig^- arg^-	8/dLB spore clone	N. Hansen
N4L [3]	mig^- lys^-	8/dLB spore clone	N. Hansen
3/dT	mig^-		M. Lubbers
THX2A [2]	mig^- ade^-	3/dT spore clone	N. Hansen
7/a	mig^-		M. Lubbers
TVA [4]	mig^- arg^-		N. Hansen
JKL [4]	mig^- lys^-		N. Hansen
DBL [4]	mig^- lys^-		N. Hansen
HBL [4]	mig^- lys^-		N. Hansen
UV01 [5]	mig^+ [6]	442 mutant	N. Hansen
UV02	mig^+ arg^-	442 mutant	N. Hansen
UV03	mig^+ arg^-	442 mutant	N. Hansen
UV04	mig^+ arg^-	442 mutant	N. Hansen
UV05	mig^+ his^-	442 mutant	N. Hansen
UV07	mig^+ ade^-	442 mutant	N. Hansen
UV08	mig^+ lys^-	442 mutant	N. Hansen
UV09	mig^+ lys^-	442 mutant	N. Hansen
Escherichia coli			
DH I			

[1] mig^+ = malate independent growth (wild type), mig^-= malate-dependent mutants.
[2] spore clone from a *S. pombe* - *S. malidevorans* mating (see Table 6).
[3] spore clone from a *S. malidevorans* - *S. malidevorans* mating (see Table 6).
[4] created by UV mutagenesis of either 442 arg^- or 442 lys^-.
[5] UV01 to UV09 were created by UV mutagenesis of 442.
[6] Uncharacterised auxotroph.

PLASMID LIST

Plasmids With Genes on Chromosome I

pFMV1 : 7.6 kb; consists of the vector pBR322 sequence and a 3.2 kb Cla I fragment of the gene *mei 2*. Shimoda *et al* , 1987.

pUC(Nuc 2)2-2 : 3.8 kb; consists of the vector pUC 19 sequence and a 1.1 kb Sal I-Hind III fragment of the gene *nuc 2*. Hirano *et al* , 1988.

pCDC 25-S9 : 8.2 kb; consists of the vector pUC 12 sequence and a 5.5 kb Sal I-Sst I fragment of the gene *cdc 25*. Russell and Nurse, 1986.

Plasmids With Genes on Chromosome II

pHE11 : 5.3 kb; consists of the vector pUC 12 sequence and a 2.6kb Eco RI-Hind III fragment of the gene *cdc 10*. Aves *et al* , 1985.

pUC 18(Top 1) : 7.8 kb; consists of the vector pUC 18 sequence and a 5.1 kb Eco RI fragment of the gene *top 1*. Uemura *et al* , 1987.

pCDC 2 : 15.1 kb; consists of the vector pDB248X sequence and a 4.7 kb Hind III fragment of the gene *cdc 2*. Durkacz *et al* , 1985.

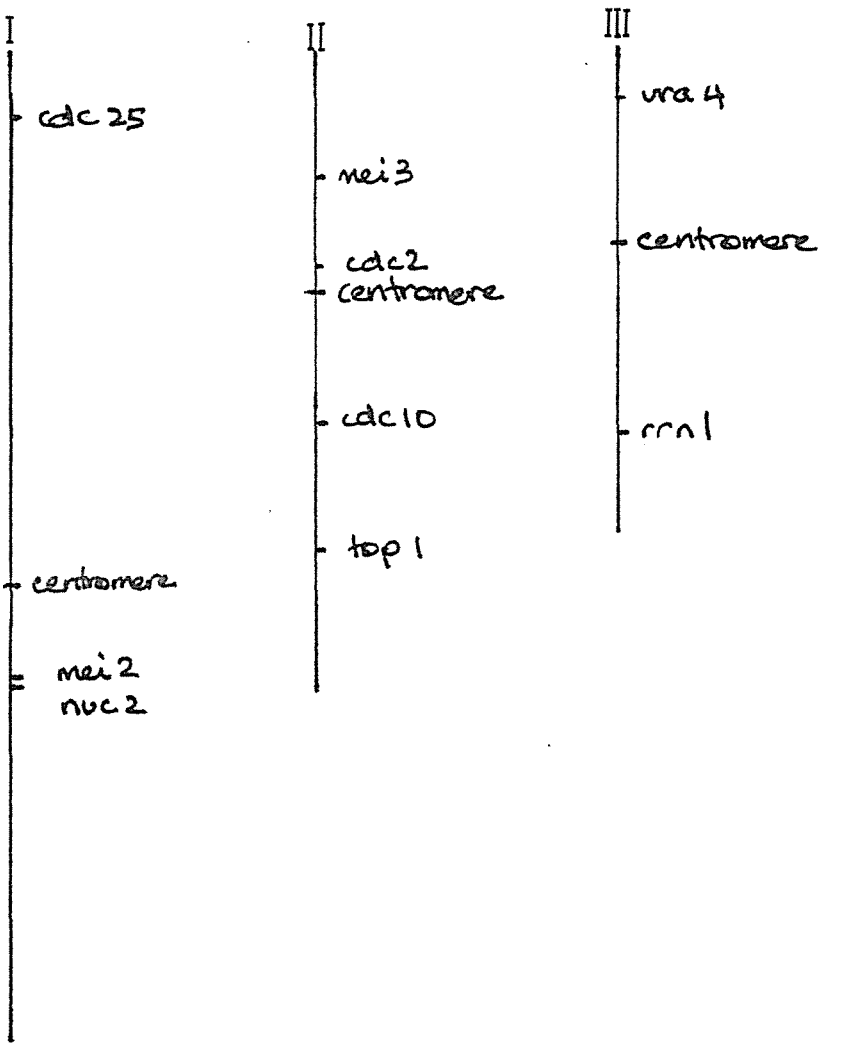
pDB(mei 3)2 : 12.7 kb; consists of the vector pDB248 sequence and a 2.6kb Hind III fragment of the gene *mei 3*. Shimoda and Uehira, 1985.

Plasmids With Genes on Chromosome III

pURA 4 : 7.3 kb; consists of the 1.8 kb *ura 4* gene in a bacteria-yeast shuttle vector.

rrn 1 : 5S rDNA gene Toda *et al*, 1984.

Fig. 2 Chromosome positions of the gene probes.



2.2. MEDIA

Media were prepared as below and, unless otherwise stated, the pH was adjusted with KOH or HCl and autoclaved at 121° C for 15 minutes. L-malic acid was added for growth of *S. malidevorans* malate-dependent mutants. The media was also supplemented, as required, with concentrations of 50 µg/ml adenine, uracil or amino acids. Weight/volume (w/v) ratios refer to g/100 ml.

Indicator Agar (IND)

0.67%(w/v) Difco yeast nitrogen base with out amino acids, 15%(w/v) glucose, 0.3%(w/v) L-malic acid, 0.22 ml 1%(w/v) Difco Bacto bromocresol green /100 ml, 3%(w/v) Davis agar, pH to 4.8.

Indicator agar identifies yeasts which utilise malic acid on the basis of colony colour. Wild type *S. malidevorans* produces blue-green colonies and *S. pombe* produces dark green colonies, neither change the colour of the surrounding media. *S. malidevorans* mig⁻ mutants produce blue colonies and change the surrounding media blue.

Malt Extract Agar +Malate (MEA/M)

3%(w/v) Difco malt extract, 1%(w/v) L-malic acid, 1%(w/v) glucose, 2%(w/v) Davis agar, pH to 4.8.

This medium is used for mating and sporulating yeasts as it encourages zygote and asci formation. It also allows vegetative growth and checks *S. pombe* strains for heterothallism. Homothallic, but not heterothallic, strains can conjugate and sporulate, on this medium.

Minimal Media (MM)

0.7%(w/v) Difco yeast nitrogen base w/o amino acids, 2%(w/v) glucose, 3%(w/v) Davis agar, pH to 4.5.

MM is used to select for yeasts with no auxotrophic requirements.

Minimal Media + Malate (MM + Mal)

As for MM with the addition of 0.3%(w/v) L-malic acid.

Minimal Malate Glucose + asparagine (MMG + asn)

0.7%(w/v) Difco yeast nitrogen base w/o amino acids, 10%(w/v) glucose, 0.3%(w/v) L-malic acid, 0.02%(w/v) asparagine, pH to 3.0.

This is the broth for fermentation cultures of *S. malidevorans* . Asparagine is added to prevent cell lysis in late exponential phase.

MYGP

0.3%(w/v) Difco malt extract, 0.3%(w/v) Difco yeast extract, 0.5%(w/v) Difco peptone, 1%(w/v) glucose, 3%(w/v) Davis agar, pH to 4.8.

MYGP is a complete medium supporting both vegetative growth and sporulation. It is used to check for reversion of *S. malidevorans* mig$^-$ mutants to mig$^+$.

MYGP/M

0.3%(w/v) Difco malt extract, 0.3%(w/v) Difco yeast extract, 0.5%(w/v) Difco peptone, 1%(w/v) glucose, 3%(w/v) Davis agar, 0.3%(w/v) L-malic acid, pH to 4.8.

As for MYGP with the addition of malate allowing growth of *S. malidevorans* mig$^-$ mutants.

Sporulation agar + malate (SPA/M)

0.7%(w/v) Difco yeast nitrogen base w/o amino acids and $(NH_4)_2SO_4$, 1%(w/v) glucose, 0.3%(w/v) L-malic acid, 3%(w/v) Davis agar, pH to 4.8.

SPA/M promotes copulation and sporulation in yeast strains. It does not contain a nitrogen source and therefore does not support vegetative growth.

Yeast Extract Agar + malate (YEA/M)

0.5%(w/v) Difco yeast extract, 3%(w/v) glucose, 0.3%(w/v) L-malic acid, 3%(w/v) Davis agar, pH to 4.8.

YEA/M supports vegetative growth but not sporulation.

Addition of antibiotics: Ampicillin (Amp) was added at a concentration of 100 µg/ml. Both liquid media and agar was allowed to cool to 60° C before the addition of ampicillin.

Luria Broth (LB)

1%(w/v) BBL trypticase peptone, 0.5%(w/v) BBL yeast extract, 0.5%(w/v) NaCl, 1.5%(w/v) Davis agar, pH to 7.3. LB is used for the growth of *E.coli*.

2.3. SOLUTIONS

Methylene blue

0.01%(w/v) methylene blue, 2%(w/v) sodium-citrate, dihydrate. Methylene blue was dissolved in 10 ml distilled water first then sodium-citrate was added and once dissolved the solution was filter sterilised and made up to the final volume with distilled water.

0.85% Saline

0.85%(w/v) NaCl.

This was used to mix yeast strains that were to be mated and as a mounting fluid for yeast slides.

1x SSC

150 mM NaCl, 15 mM sodium-citrate, pH to 7.0 with NaOH.

1x SSC was used to resuspend harvested spores. SSC was also used in many of the DNA methods.

ET

50 mM Na_2EDTA, 1 mM Tris-HCl, pH 8.0.

TE

10 mM Tris-HCl, 1 mM Na_2EDTA, pH 8.0.

1x TAE

40 mM Tris, 20 mM glacial acetic acid, 2 mM Na_2EDTA.

2.4. CULTURE TECHNIQUES

Storage

Yeast strains were stored on MYGP or MYGP/M slants in bijous at 4° C. All mig- yeast strains were streaked onto MYGP to check for reversion prior to use.

E.coli cells were stored in LB broth at -20° C.

Incubation Conditions

All yeast plates and liquid cultures were incubated at 30° C, except for mating and sporulation which were carried out at 25° C. Liquid cultures were incubated on a shaker at 100 rpm to disperse the cells, and plates were incubated inverted.

E.coli cells were grown at 37° C with liquid cultures incubated on a shaker at 200 rpm to disperse the cells.

2.5. METHODS

U V Mutagenesis

Cells were grown overnight to stationary phase (approximately 5 x 10^7 cells) in MYGP broth. Viable cell number was determined using methylene blue. The cells were diluted to give 1000 viable cells/plate and placed into a sterile petri dish where they were

irradiated by UV light until 90% of the cells were inactivated. The amount of UV light required to inactivate 90% of the cells was determined experimentally by UV kill curves using this same method. After exposure 100 µl of culture was plated onto IND plates. These plates were immediately placed into a light-proof box and incubated for 1 week to allow full colour development of the colonies.

SCREENING OF COLONIES

Mig$^-$ mutants were isolated as follows. The parental strains were either Spz A or Spz L. After 1 week the plates were examined for colonies which looked blue compared to the majority. These colonies were patched onto IND plates and incubated for 2-3 days. Blue colonies which turned the surrounding agar blue were streaked onto IND plates and incubated for three days. Single colonies were streaked on to IND and MYGP plates to check their malate-dependence.

Non malate-dependent UV mutants were isolated by the above method, however MYGP plates were used instead of IND plates. Mutants were determined by replica plating the MYGP plates onto MM plates. Colonies which grew on MYGP plates but not on MM plates were assumed to be mutated in some undetermined gene. These mutants were then tested on MM + MAL and MM + amino acids to prove malate-independence and to discover the auxotrophic marker for which they were mutant.

Fermentations

The method of Cambourn-Theewis (1990) was used. Test tubes containing 10 ml of MMG + asn broth were inoculated with the yeast strain and shaken overnight at 30° C to a cell density of approximately 10^7 cells/ml, as determined by methylene blue. Flasks (250 ml) containing 200 ml MMG + asn, were inoculated with 10^5 cells/ml. Fermentation traps containing 5 ml of sterile water were placed on the flasks which were shaken at 100 rpm at 25° C. Four ml samples were taken, initially at 12 hours, 18 hours, 24 hours and then every 4 hours as the cultures matured. The samples were placed in labelled bijous after determining the cell number and pH, and kept at -20° C until further analysis by enzyme assays.

Enzyme Assays

Fermentation samples were defrosted and analysed for glucose and L-malic acid content by enzyme assays. The change in absorbance of NAD to NADH was measured spectrophotometrically by UV light at 340 nm.

ENZYMATIC DETERMINATION OF GLUCOSE

The glucose content of the fermentation samples was determined by the following method. Three ml of sugar cocktail (0.5 M Trizma base (pH 7.6), 5 mM $MgCl_2$, 0.57

mg/ml ATP, 0.5 mg/ml NADP), 100 µl of sample and 0.7 U of glucose-6-phosphate dehydrogenase (Sigma) was mixed in a cuvette and the absorbance read. 0.6 U of hexokinase (Sigma) was then added, mixed and the absorbance read after 10 min. The change in absorbance (ΔAb) was determined and used to calculate the %(w/v) glucose by the following formula (Thornton, pers. comm.).

$$\%(w/v)\ \text{glucose} = \frac{\Delta Ab}{6.22} \times \frac{\text{final vol}}{\text{sample vol}} \times \text{dilution factor} \times 0.018$$

ENZYMATIC DETERMINATION OF L-MALIC ACID

The L-malic acid content of the fermentation samples was determined by the following method, as described in the L-malic acid kit supplied by Boehringer Mannheim. One ml of solution 1 (14.7 mg/ml L-glutamic acid), 200 µl NAD, 900 µl distilled water, 4 U glutamate-oxaloacetate transaminase and 100 µl of sample were added to the cuvette, mixed and the absorbance read after 3 min. The reaction was started by the addition of 60 U L-malate dehydrogenase. The solution was mixed and the absorbance read after 10 min. The ΔAb was used to calculate the amount of L-malic acid (g/L) as described in the kit.

Iodine vapour test

The iodine vapour test of Leupold (1970) was used to isolate nonsporulating colonies.

Genetic Crosses, for complementation analysis

Crosses were carried out by the standard genetical protocol of Gutz *et al* (1974). However, the mating mixture was streaked onto MM + MAL rather than MM to allow noncomplementing as well as complementing diploids to grow. A positive control cross of *S. pombe* $h^+ \times h^-$ cells was carried out with each batch of crosses.

Genetic Crosses, for auxotrophic markers

Crosses were carried out as described by Moreno *et al* (1990). Spores were harvested by random spore analysis. The mating mixture was checked for diploids at 15 hours, rather than 12 hours, under the microscope.

Random Spore Analysis

The method of Moreno *et al* (1990) was used with modifications.

The mating mixture containing lots of asci was scraped into a 50 mg/ml solution of Novozyme and incubated at 30° C for 4 hours or until the asci walls were digested. The sample was washed with water, centrifuged in an eppendorf centrifuge, resuspended in 30%(v/v) ethanol and incubated at 30° C for a further 4 hours or until no viable vegetative cells were detected under the microscope. The spore solution was then

centrifuged, washed and resuspended in 1x SSC. Varying dilutions were plated onto selective IND agar and incubated for 7 days to allow full colour development. Blue colonies were picked and patched onto MYGP/M plates. After 2 days the colonies were replica plated onto selective agar plates to identify double mutants (e.g. mig$^-$ lys$^-$ or mig$^-$ arg$^-$).

Unless otherwise stated, all steps of the following methods, were carried out at 4° C.

Preparation of Chromosomal DNA Samples For TAFE

A modification of the method of Murray (1989) for preparing chromosomal DNA from *S. pombe* and *S. malidevorans* was used.

Cells were grown to late log phase in MYGP/M broth, 3 x 10^9 cells (*S. pombe*) or 2 x 10^9 cells (*S. malidevorans*) were harvested and washed twice with chilled ET buffer. Cells were resuspended up to 2 ml in CES (20 mM sodium-citrate, 50 mM Na_2EDTA, 0.9 M sorbitol pH 7.5) containing 1040 U lyticase (Sigma) and incubated for 45 min with shaking at 37° C. Two ml of 1.2% LMP agarose in CE (20 mM sodium-citrate, 50 mM Na_2EDTA, pH 7.5) was added, mixed and the cells added to moulds. The resulting agarose plugs were removed from the moulds into 0.2 mM Na_2EDTA with 1% SDS and incubated for 2 hours at 60° C, after which time they were transferred to 5 ml NES solution (0.5 M Na_2EDTA, 1% Na-N-lauroyl sarcosine, 10 mM Tris-HCl, pH 8.0) containing 2 mg/ml proteinase K and incubated at 50° C for 24 hours. Finally the plugs were washed and stored in ET at 4° C.

TAFE Electrophoresis Conditions

These were as described by Murray (1989). Gels were 0.7% agarose (Sigma, Type 1) and run at a field strength of 2 V/cm using a switching time of 60 min for 114 hours in 3.5 L running buffer at 15° C. The running buffer was made by diluting 175 ml 20x TAFE (0.2 M Tris, 10 mM EDTA (free acid), 0.5%(v/v) glacial acetic acid) with 3.325 L distilled water, sterilised and chilled to 15 ° C. Gels were stained in ethidium bromide (2 μg/ml) for 30 min and DNA was visualised by fluorescence on a UV transilluminator before being photographed.

Blotting

DNA, from gels, was depurinated and transferred onto either nitrocellulose or Amersham's Hybond-C extra by using a Pharmacia VacuGene XL vacuum blotting system. This was done using the VacuGene XL protocol No. 1, in the VacuGene XL instruction manual. However solutions I, II, and III, were slightly different: solution I was 0.25 M HCl; solution II was 0.5 M NaCl, 0.5 M NAOH; solution III was 0.5 M

Tris-HCl, 2 M NaCl, pH to 7.4. After blotting the filters were placed between sheets of Whatman No. 1 blotting paper and baked at 80° C under vacuum for 2 hours.

Digestion of DNA Plugs With *Not* I

Agarose plugs, preparation described above, were subjected to a rigorous washing procedure similar to that of Fan *et al* (1988). This was done firstly, to remove any residual proteinase K, secondly, to remove the PMSF (phenylmethyl-sulphonyl fluoride), and thirdly to equilibrate the plugs in the digest mixture. All of the washings were done at room temperature.

The first two washings were carried out for one hour in TE containing 5.8 mg/ml PMSF in ethanol. The next three washes were for 30 min in 5 ml of running buffer (0.5x TBE: 45 mM Tris-HCl, 45 mM Boric acid, 25 mM Na_2EDTA). The final two washes were for 30 min in 0.5x TBE, 1x NEBuffer (supplied with the enzyme by New England BioLabs). The plugs were digested overnight with *Not* I in a final volume of 300 µl. After incubation, plugs were loaded into the wells of an agarose gel and run on a CHEF apparatus at 10° C, conditions shown elsewhere (Plate 5). Gels were run with Bio-Rad *S. cerevisiae* plugs as DNA size markers.

Preparation of Plasmid DNA

The plasmids were obtained from research groups overseas as ethanol precipitates of DNA. These were recovered by placing in an ethanol bath at -70° C for 30 min and centrifuging for 15 min in an Eppendorf centrifuge. The supernatant was removed and the pellet washed with 70%(v/v) ethanol and centrifuged at 4° C for 5 min. The DNA was resuspended in 10 µl TE after being dried under vacuum.

Preparation of Competent *E.coli* DH1 Cells

A 10 ml overnight starter culture of DHI was diluted 1:100 into 100 ml of LB and grown for 2-3 hours until OD_{550}= 0.4-0.6. Cells were harvested at 4,500 g for 12 min, resuspended in an equal volume of sterile cold 50 mM $CaCl_2$, left to stand for 30 min, centrifuged as above and resuspended in 1/25 volume with 50 mM $CaCl_2$.

Transformations

To 200 µl of competent cells 1 µl of plasmid DNA was added and the mixture left on ice for 20-30 min. Cells were heat shocked at 42° C for 90 seconds, placed on ice, and 900 µl of LB added (800 µl LB, 50 µl x-gal, 20 µl IPTG for plasmids containing the Lac-Z gene). The mixture was incubated for 30-60 min with shaking, the cells were plated out at dilutions of 10 µl, 50 µl, and 100 µl, onto selective antibiotic plates and grown for 16 hours.

Rapid Boils

Test tubes containing 5 ml of LB + ampicillin (100 μg/ml) were inoculated with a single bacterial colony and grown overnight. Cells were harvested in an eppendorf centrifuge and the DNA extracted by the rapid boil method of Holmes and Quigley (1981). Transformed cells were selected by restriction enzyme digestion and those cells containing the correct plasmid were stored at -20° C. The digests were run on 0.7% agarose mini gels, in 1x TAE buffer for 1 hour at 100 V. Gels were stained in ethidium bromide (2 μg/ml) and the DNA visualised by fluorescence on a UV transilluminator before being photographed.

Restriction Digests

Restriction digests were carried out using enzymes from New England BioLabs as directed by the company.

Alkaline Lysis Plasmid Preparations

Alkaline lysis plasmid preparations were carried out by a modified method of Maniatis *et al* (1982). After the transformants were checked by rapid boils, the cells were diluted 1:100 into 250 ml LB + ampicillin (100 μg/ml) and grown for 3-5 hours with shaking. Once the cells had grown the plasmid was amplified with chloramphenicol at a concentration of 150 μg/ml, and continued incubation overnight. Cells were harvested by centrifugation (10,000 g, 5 min) and resuspended in TE then centrifuged again (10,000 g, 5 min). The supernatant was removed and the pellet resuspended in 8 ml glucose solution (50 mM glucose, 25 mM Tris pH 8.0, 10 mM Na_2EDTA). One ml of fresh lysozyme (50 mg/ml in glucose solution) was added and the cells were left for 30 min, after which 10 ml of fresh NaOH-SDS solution (0.2N NaOH, 1% SDS) was added. The solution was gently mixed by inversion and left for 20 min. The addition of 8.9 ml fresh potassium acetate solution (60 ml 5M KOAc, 11.5 ml glacial acetic acid made up to 100 ml) was followed by centrifugation at 27,000 g for 1 hour to precipitate proteins. The supernatant was decanted into 12 ml of isopropanol, mixed and left for 1 hour. The DNA was pelleted at 2000 g for 30 min, washed in 20 ml of 95% ethanol (2000 g, 20 min), and air dried for 5-10 min. The DNA was dissolved in 9 ml TE and 1.08 g CsCl added per ml DNA solution and 0.9 ml of 5 mg/ml ethidium bromide added.

Samples were centrifuged in an ultracentrifuge at 400,000 g, 15° C for 5 hours. The band was visualised with long wave UV light and extracted from the sample with syringes (Maniatis, 1982). Ethidium bromide was extracted using water saturated isobutanol until the DNA solution was clear, then once more. Samples were dialysed against three changes of sterile TE (500-1000 volumes). Samples were finally ethanol precipitated, resuspended in TE and the DNA concentration determined spectrophotometrically at 260/280 nm prior to storage at 4° C in NUNC tubes.

Ethanol Precipitation

DNA was concentrated by ethanol precipitation as described in Maniatis *et al* , 1982.

Removal of Plasmid Inserts From Agarose

In the case of certain plasmids which contain yeast sequences other than the gene of interest, the insert had to be cut out of the vector. DNA (4 µg insert DNA) was cut, in a volume of 75 µl, with restriction enzymes and 1 µl was checked for complete digestion on a mini gel. The cut mixture was concentrated by ethanol precipitation and run in 1 well of a 1% sea plaque gel in 1x TAE at 100 V. The gel was stained in ethidium bromide, and the bands visualised under long wave UV light to allow the insert band to be cut out of the gel. The insert was placed on top of approximately 5 mm of siliconised glass wool in a 500 µl eppendorf tube which had a hole in the bottom. This tube was centrifuged at 6500 rpm inside a 1.5 ml eppendorf tube for 10 min to allow the DNA to move through the glass wool and into the bottom tube (Technical Tips, 1990). The DNA was quantitated by electrophoresing 1 µl on a gel against standards. The recovery was approximately 20%.

Synthesis of DNA Probes

Approximately 200 ng of DNA was labelled with [α ^{32}P]-dCTP by random priming (Taylor, J.M. *et al*, 1976; Whitfield, P.L. *et al*, 1982) using mini-spin columns.

Preparation of mini-spin columns was as follows. Sterile Sephadex G80 was washed twice with TEN (TE with 0.1 M NaCl) and centrifuged in an MSE bench centrifuge at speed 10 for 3 min. A 1 ml syringe plugged with siliconised glass wool, was filled to the top with washed Sephadex and centrifuged as above at speed 3 for 3 min. The packed volume was 0.8 ml and 200 µl of TEN was then added. (Before use, the column was centrifuged as before at speed 3 for 3 min). Once the probe had been incubated for 30 min,160 µl TEN was added and the probe (approximately 200 µl) was pipetted onto the column. The column was then centrifuged at speed 3 for 3 min. The probe was collected in an eppendorf tube at the base of the column. 200 µl of TEN and 200 µl of herring sperm DNA was added to this and the probe was boiled for 2 min before addition to the prehybridised filters, along with hybridisation solution.

Hybridisation

Filters were prehybridised in roller tubes with 10x Denharts (0.2%(w/v) ficoll 400, 0.2%(w/v) polyvinylpyrolidone, 0.2%(w/v) bovine serum albumin) in 6x SSC for 4 hours at 68° C. Hybridisation was carried out in the same roller tube in hybridisation solution (1 M NaCl, 50 mM NaH_2PO_4, pH 6.5, 2 mM Na_2EDTA, 0.5% SDS, 10x Denharts) overnight at 68° C. Following hybridisation the filters were washed 4 times at 68° C for 20-30 min. The first two washes were in 2x SSC + 0.1% SDS and the last

two washes in 1x SSC. Filters were put into a cassette with x-ray film at -70° C and exposed for variable periods of time (overnight to several days). Autoradiographs were developed either by hand or by an autodeveloper.

3. RESULTS

3.1. THE MUTANT CHARACTERISTIC

In this study several mutants were isolated by UV mutagenesis. The additional mig$^-$ mutants were isolated for use in the complementation tests so that all possible complementation groups would be represented. The mig$^+$ auxotrophic mutants were isolated so that their chromosome patterns could be compared to the mig$^-$ mutants. The fermentation patterns of Lubbers's (1987) four mig$^-$ mutants were determined and compared to that of #11.

3.1.1. U V Mutagenesis

Several *S. malidevorans* 442 strains were subjected to enough UV light to allow a 90% inactivation rate. From UV kill curves, the exposure time for 90% inactivation was determined to be an average time of 94 seconds. Individual irradiated colonies were picked and patched onto selective media to determine mutants.

A total of 4 mig$^-$ mutants and 8 mig$^+$ auxotrophic mutants were isolated (see strain list). Irradiation of Spz L gave 3 malate-dependent mutants with a mutation rate of 1/3000 colonies. Irradiation of Spz A gave 1 malate-dependent mutant with a mutation rate of 1/1700 colonies. The 8 mig$^+$ mutants were obtained from irradiation of wild type 442, giving a mutation rate of 1/175 colonies.

3.1.2. Enzyme Analysis of Fermentation Trials

Fermentation trials, in MMG + asn, were carried out on five mig$^-$ strains: #11, 3/dLB, 8/dLB, 3/dT, and 7/a. The fermentation samples were analysed enzymatically to determine both the glucose and L-malic acid content present at various intervals. Duplicate trials of each culture were carried out and the samples were analysed enzymatically two to three times. The results are shown in Figs.3-7 and Tables 1-5. As cell growth varies between cultures, the samples for enzyme analysis were chosen based on similar cell number not on similar time interval. Therefore the strains were at the same stage in their growth pattern.

From the results, there does not appear to be any significant difference between the five mig$^-$ mutants. All mig$^-$ mutants reached a max. cell number of approx. 3 x 10^7, used very little glucose, at most 25%, and essentially all of the L-malic acid by a similar stage of cell growth. Minor variations between duplicate cultures can be explained due to differing growth rates, as one culture can lag behind the other.

Fig. 3. Fermentation trial of the mig⁻ mutant #11.

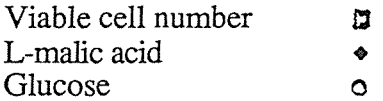
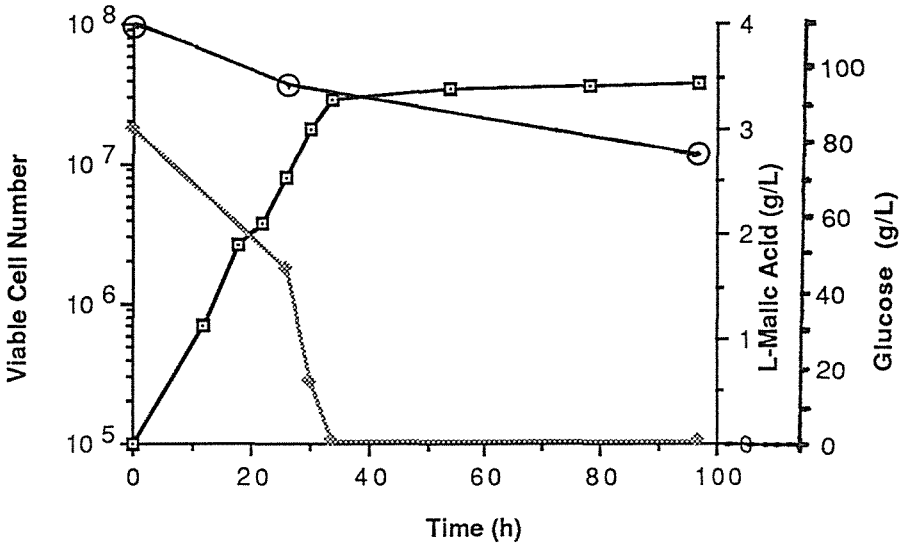


Table 1. Fermentation trial of the mig⁻ mutant #11.



Time (h)	Culture Number	Viable cells/ml	Glucose (g/L)	%Glucose Utilisation	L-malate (g/L)	%L-Malate Utilisation
0	1	1.0 x 10^5	104.5	0	3.17	0
	2	1.0 x 10^5	104.5	0	3.17	0
26	1	9.8 x 10^6	82.2	20.0	1.35	57.4
	2	6.2 x 10^6	104.5	0	1.99	37.2
30	1	2.2 x 10^7			0.367	88.4
	2	1.3 x 10^7			0.812	74.4
34	1	3.3 x 10^7			0.0213	99.3
	2	2.2 x 10^7			0.0520	98.4
97	1	4.0 x 10^7	69.3	32.6	0.0197	99.4
	2	3.3 x 10^7	85.0	17.3	0.0344	98.9

Fig. 4. Fermentation trial of the mig⁻ mutant 3/dLB.

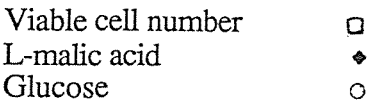
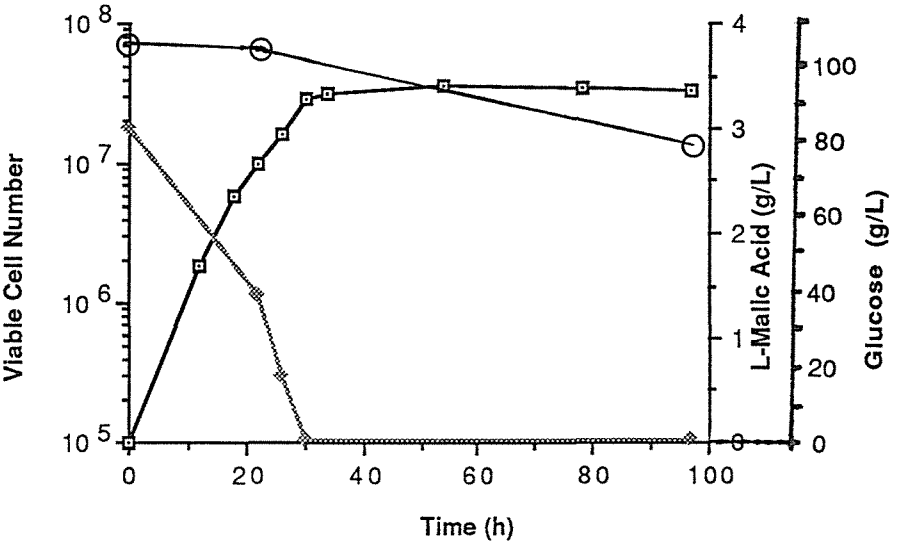


Table 2. Fermentation trial of the mig⁻ mutant 3/dLB.



Time (h)	Culture Number	Viable cells/ml	Glucose (g/L)	%Glucose Utilisation	L-malate (g/L)	%L-Malate Utilisation
0	1	1.0 x 10^5	104.5	0	3.17	0
	2	1.0 x 10^5	104.5	0	3.17	0
22	1	9.1 x 10^6	104.5	0	1.64	48.3
	2	1.1 x 10^7	99.5	3.2	1.20	62.1
26	1	1.6 x 10^7			0.7090	77.6
	2	1.6 x 10^7			0.5910	81.4
30	1	9.1 x 10^7			0.0236	99.3
	2	9.1 x 10^7			0.0248	99.2
97	1	3.3 x 10^7	73.4	28.6	0.0184	99.4
	2	3.3 x 10^7	83.6	18.7	0.0223	99.3

Fig. 5. Fermentation trial of the mig⁻ mutant 8/dLB.

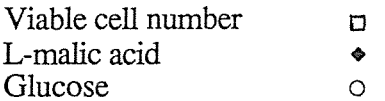
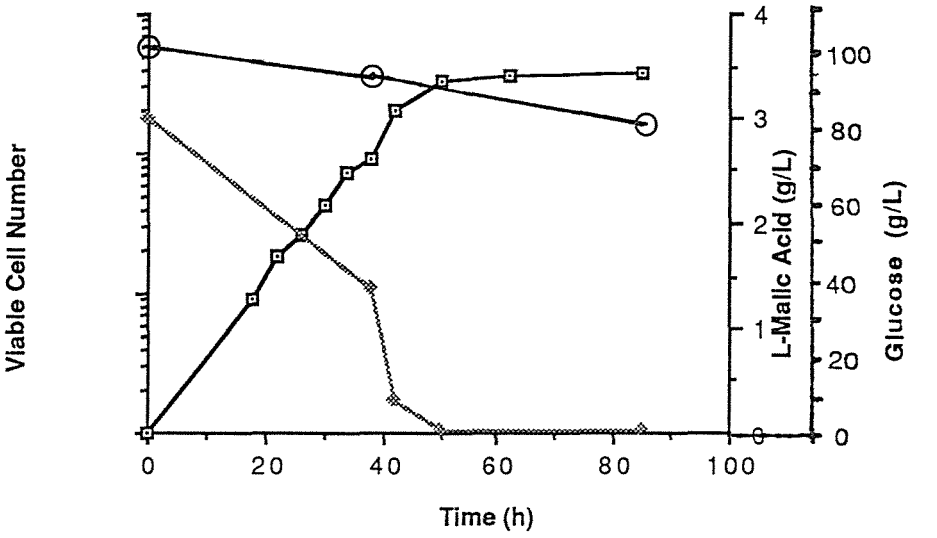


Table 3. Fermentation trial of the mig⁻ mutant 8/dLB.

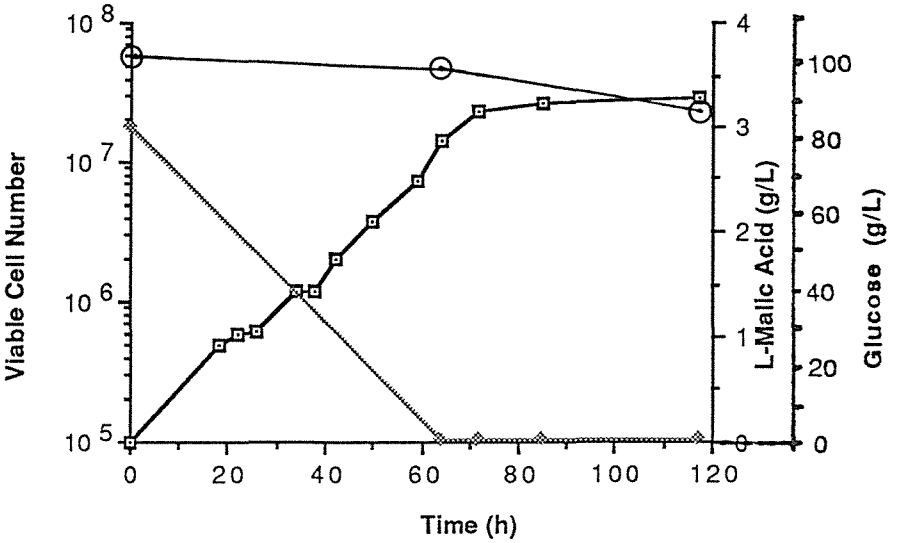


Time (h)	Culture Number	Viable cells/ml	Glucose (g/L)	%Glucose Utilisation	L-malate (g/L)	%L-Malate Utilisation
0	1	1.0×10^5	104.5	0	3.17	0
	2	1.0×10^5	104.5	0	3.17	0
38	1	1.1×10^7	101.5	1.3	1.53	51.7
	2	8.5×10^6	87.5	14.9	1.24	60.9
42	1	1.5×10^7			0.378	88.1
	2	2.5×10^7			0.225	92.9
50	1	2.9×10^7			0.0236	99.3
	2	3.7×10^7			0.0095	99.7
85	1	3.3×10^7	76.6	25.5	0.0244	99.2
	2	4.1×10^7	86.1	16.2	0.0110	99.7

Fig. 6. Fermentation trial of the mig⁻ mutant 3/dT.

Viable cell number	□
L-malic acid	◆
Glucose	○

Table 4. Fermentation trial of the mig⁻ mutant 3/dT.

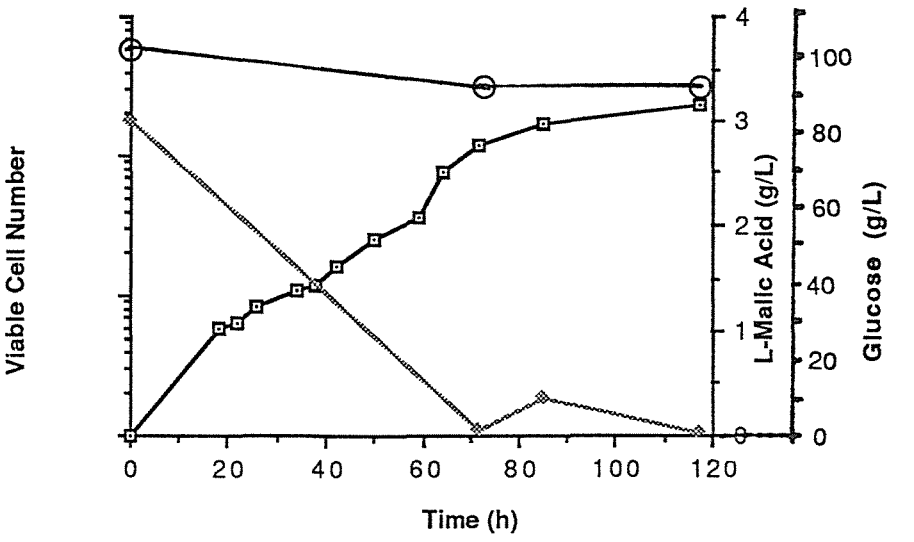


Time (h)	Culture Number	Viable cells/ml	Glucose (g/L)	%Glucose Utilisation	L-malate (g/L)	%L-Malate Utilisation
0	1	1.0 x 10^5	104.5	0	3.17	0
	2	1.0 x 10^5	104.5	0	3.17	0
64	1	1.7 x 10^7	95.4	7.2	0.0085	99.7
	2	9.1 x 10^6	100.4	2.3	0.0179	99.4
72	1	2.9 x 10^7			0.0095	99.7
	2	1.7 x 10^7			0.0291	99.1
85	1	2.7 x 10^7			0.0236	99.3
	2	2.4 x 10^7			0.6520	79.4
117	1	2.5 x 10^7	85.7	16.7	0.0177	99.4
	2	2.3 x 10^7	86.6	15.8	0.0273	99.1

Fig. 7. Fermentation trial of the mig⁻ mutant 7/a.

Viable cell number	□
L-malic acid	◆
Glucose	○

Table 5. Fermentation trial of the mig⁻ mutant 7/a.



Time (h)	Culture Number	Viable cells/ml	Glucose (g/L)	%Glucose Utilisation	L-malate (g/L)	%L-Malate Utilisation
0	1	1.0×10^5	104.5	0	3.17	0
	2	1.0×10^5	104.5	0	3.17	0
72	1	1.2×10^7	93.6	8.9	0.0730	97.7
	2	1.1×10^7	91.4	11.1	0.0118	99.6
85	1	1.4×10^7			0.4510	85.8
	2	1.9×10^7			0.2820	91.1
117	1	2.4×10^7	96.1	6.5	0.0174	99.5
	2	2.1×10^7	90.0	12.4	0.0377	98.8

3.2. COMPLEMENTATION TESTS

Before complementation tests could be carried out other selectable markers had to be put into the mig$^-$ mutants by genetic crossing. These markers were used to select for diploids which were then tested for complementation of mig$^-$.

3.2.1. Auxotrophic Markers

The method of Gutz *et al* (1974) was used for *S. pombe* - *S. malidevorans* crosses. Diploids were selected for on MM, sporulated and the spores screened for double mutants. In excess of 200 spores were tested per cross. The results are tabulated in Table 6 a. The 3/dLB crosses gave all four expected progeny classes, however the cross with 131 gave two additional classes which were mig$^+$ or mig$^-$ but deficient in some other unknown auxotrophic marker. All of the diploids analysed from the ten separate 8/dLB crosses with Spk 3 gave prototrophic spore clones only, as did the diploids from the four 3/dT x Spk 3 crosses. Both of the adenine classes were found in the 3/dT x Spk 2 cross. However as only red ade$^-$ colonies were selected further, analysis is incomplete. On further testing, 14 of these colonies proved to be ade$^+$. Several crosses were also done where 8/dLB was crossed with 131. All of the spore clones in these crosses were prototrophic. Any crosses involving 7/a yielded either no mating or spore clones that were all prototrophic.

The method of Moreno *et al* (1990), where strains were mated on MEA/M plates and the spores taken directly from these plates, was used in *S. malidevorans* -*S. malidevorans* crosses (Table 6 b). The mutants were selected for on IND plates, however, mig$^+$ spore clones were not analysed for their auxotrophic markers. Several double mutants were obtained in all strains except 7/a and 3/dT. The number of blue (malate-dependent) colonies was very low as was the number of double mutants.

3.2.2. Complementation Tests

Crosses of strains were carried out by the method of Gutz *et al* (1974), but diploids were selected for on MM + MAL plates. Individual diploid colonies were patched onto MM + MAL plates after a 15 hour incubation and analysed for malate-dependence (Table 7). All of the crosses were repeated at least twice. The results in Table 7 are combined for all of the crosses. The 8/dLB x HBL cross was repeated three times but only one of the crosses produced any diploid colonies giving the low number reported (five).

Most of the crosses show a mixture of mig$^+$ and mig$^-$ diploids, which was unexpected. To determine that "true diploids" were obtained and not spore clones, one or more colonies from

Table 6. Spore clone analysis of matings between mig$^-$ and auxotrophic mig$^+$ strains.

a *S. pombe* - *S. malidevorans* crosses.

b *S. malidevorans* - *S. malidevorans* crosses.

The genotype of the spore clones is shown where "m" = mig and "x" = auxotrophic marker. The spore clones analysed for each cross in this table were from one independent cross.

a Cross	Number of Spore Clones						Total
	m^-x^+	m^-x^-	m^+x^+	m^+x^-	m^-	m^+	clones
3/dLB x Spk 3	80	35	17	89	-	-	221
3/dLB x 131	105	36	52	54	6 [1]	93 [1]	346
8/dLB x Spk 3	0	0	200	0	-	-	200
3/dT x Spk 3	0	0	200	0	-	-	200
3/dT x Spk 2	1	4	13	80	-	-	98
b Cross	m^-x^+	m^-x^-	m^+				Total
8/dLB x Spz A	111	18	700				828
8/dLB x Spz L	17	8	225				250
3/dLB x Spz A	13	2	150				165
3/dLB x Spz L	60	1	411				472
3/dT x Spz A	5	0	295				300
3/dT x Spz L	14	0	88				102
7/a x Spz A	1	0	179				180
7/a x Spz L	0	0	200				200

[1] Colonies were "-" for some auxotroph other than leu.

Table 7. Complementation analysis of mig$^-$ strains.

The diploids from the complementation crosses were tested for malate-dependence. A number of mig$^-$ and mig$^+$ colonies were obtained from each complementation test. "-" = mig$^-$, "+" = mig$^+$, ND = not done, "*" = crosses which were subjected to spore clone analysis.

Strains	TVA	HBL	DBL	JKL	3/dT	8/dLB	3/dLB	#11
#11	56- 29+ *	38- 14+ *	7- 46+ *	57- 16+ *	ND	100- 65+ *	75- 29+	-
3/dLB	0- 24+	12- 34+ *	17- 26+	7- 18+ *	14- 41+	0- 21+	-	-
8/dLB	2- 87+ *	0- 5+ *	1- 48+ *	5- 32+ *	4- 58+ *	0- 100+	-	-
3/dT	2- 91+ *	1- 10+	0- 117+ *	4- 17+ *	-	-	-	-
JKL	3- 49+ *	ND	-	-	-	-	-	-
DBL	19- 87+	ND	-	-	-	-	-	-
HBL	11- 20+ *	-	-	-	-	-	-	-
TVA	-	-	-	-	-	-	-	-

each of the crosses with an "*", in Table 7, were sporulated and around 50 of the spores analysed by random spore analysis (Table 8). Where possible a mig$^+$ and mig$^-$ diploid from the same cross were sporulated.

Because of the presence of large numbers of mig$^+$ and mig$^-$ diploids in each cross, a control mating was carried out with 8/dLB x 8/dLB. All of the diploids were expected to be mig$^-$ (no complementation). However, all of the diploids tested were mig$^+$, suggesting that there is some other factor influencing their mig phenotype.

Analysis of the spore clones showed that some of the colonies isolated from the crosses were true diploids (since there was good segregation of the spores into classes) and some of the colonies were actually spore clones (because of a lack of segregation when sporulated), also some of the crosses had a mixture of both. It should be noted that in most of the spore clones the arg$^-$ or ade$^-$ classes are missing or under-represented.

The crosses with an "*" in Table 8 were analysed further. The mig$^-$ clones were mated with both of the parents to try to determine if complementation had actually occurred (Table 9). The mated mixture was streaked onto MM or MM + amino acids as there were no auxotrophic markers in many of the mig$^-$ spore clones. If complementation had occurred, the mig$^-$ clones would be expected to mate and form mig$^+$ diploids with either parent or neither parent. If there originally had been no complementation the mig$^-$ clones would mate and form mig$^+$ diploids with neither parent.

The #11 x DBL (mig$^+$ diploid) and 8/dLB x 3/dLB crosses gave the segregation of the mig$^-$ clones that is expected for complementing strains, although there was an unexpected class where the spore clones complemented with both parents. In the mig$^+$ diploid of #11 x DBL, 6 spore clones complemented with #11, 1 clone complemented with DBL, no clones mated to give mig$^+$ with neither parent and 11 complemented with both. In 8/dLB x 3/dLB, 5 clones mated to give mig$^+$ with 8/dLB, 10 with 3/dLB, 5 with neither parent and 13 complemented with both. The remaining crosses gave results that did not indicate that complementation had occurred. The two mig$^-$ spores from the mig$^-$ diploid of #11 x DBL complemented with both parents. In the #11 x JKL cross, 18 of the 20 clones mated to give mig$^+$ with both parents.

3.3. CHROMOSOME ANALYSIS

3.3.1. Chromosomal Banding Patterns on TAFE

Yeast cells were protoplasted and embedded in agarose before lysing to ensure minimal breakage of chromosomal DNA. These plugs were run on TAFE under the conditions described in the materials and methods.

Table 8. Spore clone analysis of matings between mig$^-$ strains.

These spore clones were from colonies from the matings marked "*" in Table 7. The genotype of the spore clones is shown with a "+" or "-" where m = mig, a = ade or arg and l = lys. An "*" indicates that the mig$^-$ spore clones were analysedfurther by mating with the parents. "mig of diploid" refers to the mig phenotype of the colony from Table 7 that was sporulated.

Cross	mig of diploid	Number of Spore Clones						
		$m^+a^+l^+$	$m^-a^-l^-$	$m^+a^+l^-$	$m^-a^+l^+$	$m^+a^-l^-$	$m^-a^-l^+$	$m^+a^-l^+$
3/dT x JKL	+	52	0	0	0	0	0	0
	+	16	3	8	9	6	2	8
	-	24	3	20	4	1	0	0
3/dT x DBL	+	52	0	0	0	0	0	0
	+	52	0	0	0	0	0	0
	+	40	0	0	3	0	0	9
3/dT x TVA	+	52	0	0	0	0	0	0
	-	0	1	1	0	0	0	0
3/dT x 8/dLB	+	52	0	0	0	0	0	0
	+	52	0	0	0	0	0	0
	+	52	0	0	0	0	0	0
	-	2	1	1	25	0	0	0
TVA x JKL	+	26	3	20	2	0	0	1
	-	0	0	0	52	0	0	0

Table 8. con't.

Cross	mig of diploid	Number of Spore Clones						
		$m^+a^+l^+$	$m^-a^-l^-$	$m^+a^+l^-$	$m^-a^+l^+$	$m^+a^-l^-$	$m^-a^-l^+$	$m^+a^-l^+$
TVA x HBL	+	27	3	14	8	0	0	0
	-	1	0	0	51	0	0	0
8/dLB x TVA	+	52	0	0	0	0	0	0
	+	52	0	0	0	0	0	0
	-	0	0	0	52	0	0	0
8/dLB x JKL	+	22	4	11	5	9	0	1
	-	3	5	0	40	0	0	1
8/dLB x DBL	-	1	0	0	25	26	0	0
8/dLB x HBL	+	52	0	0	0	0	0	0
8/dLB x 3/dLB	+*	49	108	13	146	104	0	187
8/dLB x #11	+	48	0	0	0	0	0	4
	-	0	0	0	52	0	0	0
3/dLB x HBL	+	12	5	10	12	4	0	4
	-	0	0	0	52	0	0	0

Table 8. con't.

Cross	mig of diploid	Number of Spore Clones						
		$m^+a^+l^+$	$m^-a^-l^-$	$m^+a^+l^-$	$m^-a^+l^+$	$m^+a^-l^-$	$m^-a^-l^+$	$m^+a^-l^+$
3/dLB x JKL	+	20	1	16	9	0	0	0
	-	0	0	0	52	0	0	0
#11 x HBL	+	52	0	0	0	0	0	0
	-	7	3	20	18	9	0	1
#11 x DBL	+*	9	0	31	18	0	0	0
	-*	0	0	0	44	0	0	0
#11 x TVA	+	52	0	0	0	0	0	0
	+	52	0	0	0	0	0	0
	-	0	0	0	36	0	0	0
	-	47	0	0	5	0	0	0
#11 x JKL	+*	15	18	15	13	0	0	0
	-	5	3	0	67	1	0	0

Table 9. Mating analysis of mig$^-$ spore clones with parent strains.

The mig$^-$ spore clones with an "*" in Table 8 were mated with the parent strains. "mig of diploid" refers to the mig phenotype of the original colony (Table 7) and "spore genotype" refers to mig$^-$ spores from Table 8.

P_1 and P_2 refer to the first and second parents respectively.

$P_1^-P_2^+$ = strains that complemented with the second parent only;

$P_1^+P_2^-$ = strains that complemented with the first parent only;

$P_1^-P_2^-$ = strains that did not complement with the either parent;

$P_1^+P_2^+$ = strains that complemented with both parents.

Cross P_1 x P_2	mig of Diploid	Spore Genotype	Number Spores			
			$P_1^-P_2^+$	$P_1^+P_2^-$	$P_1^-P_2^-$	$P_1^+P_2^+$
#11 x JKL	+	-++	0	2	0	10
		---	0	0	0	8
#11 x DBL	+	-++	1	6	0	11
	-	-++	0	0	0	2
8/dLB x 3/dLB	+	---	10	5	5	13

S. malidevorans 442 wild type has a similar chromosomal banding pattern to *S. pombe* on TAFE, which confirms earlier findings (Murray,1989). This has also been seen on CHEF. The chromosomal banding pattern of the eight *S. malidevorans* mig$^+$ auxotrophic mutants was also similar to the wild type (Plate 1), whereas the ten *S. malidevorans* mig$^-$ mutants and spore clones had differing banding patterns. The banding patterns of all of the strains run on TAFE can be represented by four differing patterns (Fig. 8), three of which show that the middle chromosome (shown to be chromosome II by probing) has a changed mobility.

Pattern 1 is the wild type pattern that was seen in all of the mig$^+$ strains, this pattern was seen consistently in only one mig$^-$ mutant (JKL). Pattern 2 shows the altered chromosome II pattern seen by Murray (1989) for #11. It was seen in the #11 ade$^-$ spore clone, XL1A, 8/dLB and in #11 occasionally. Pattern 3 was seen in three of the four mig$^-$ mutants isolated in this study (DBL, HBL and TVA). It was observed for the spore clones (N1A and THX2A) of 3/dLB and 3/dT (not shown). Pattern 4 shows only two bands as chromosomes I and II appear to be comigrating. This was seen in 3/dLB and in most of the #11 plugs in this study.

In this study, the three banded pattern (2) was seen consistently in 8/dLB and the spore clone XL1A but only occasionally in the #11 mutant (Plate 1). It was seen in one preparation of #11 plugs and then only once those plugs were several months old. A changed banding pattern was also seen in two of the three mig$^-$lys$^-$ strains DBL and HBL. Originally they all gave the wild type pattern (1) but seven months after isolation, the patterns of DBL and HBL changed to pattern 3 while JKL stayed as wild type. These patterns were constant for the rest of this study.

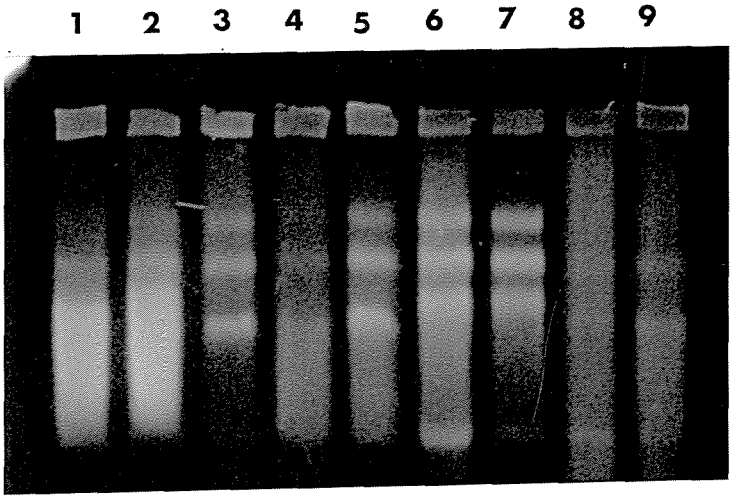
All of these patterns were reproducible as they were seen in at least two independent preparations of plugs and on more than four runs. The altered pattern was only seen in the mig$^-$ mutants. The strains 3/dT and 7/a were not characterised on TAFE as it proved difficult to obtain good preparations.

Chromosome III of the *S. malidevorans* yeasts appears to have two possible positions with the mobility either the same or slightly lower than chromosome III of 972. This variation was seen in most of the *S. malidevorans* strains studied. It occurred in different plug preparations of the same strain and was always the same in different runs of the same plug preparation. This indicates that the chromosome III variation is normal and not caused by changes in chromosome size. The lowest band in the photos was thought to be due to degradation products of all three chromosomes.

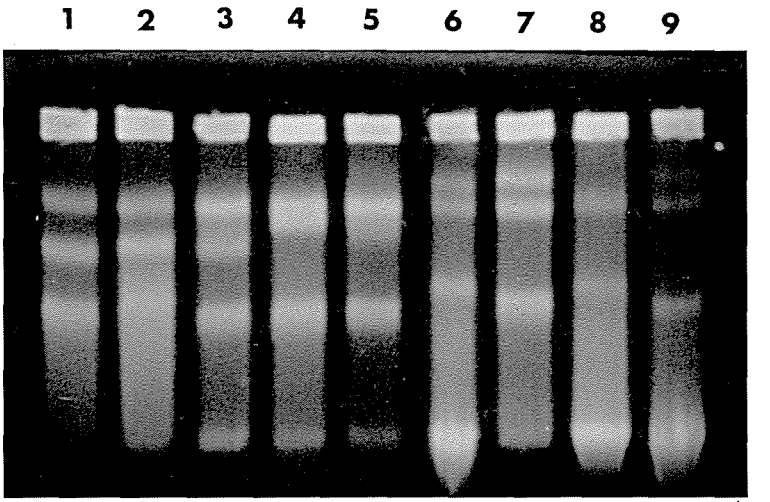
Plate 1. Chromosomal banding patterns on TAFE.

a *S. malidevorans* mig^+ auxotrophic mutants.
Lane 1. 442, Lane 2. UV01, Lane 3. UV02, Lane 4. UV03, Lane 5. UV04, Lane 6. UV05, Lane 7. UV07, Lane 8. UV08, Lane 9. UV09.

b *S. malidevorans* mig^- mutants and wild types.
Lane 1. 972, Lane 2. 442, Lane 3. JKL, Lane 4. DBL, Lane 5. HBL, Lane 6. 8/dLB, Lane 7. XL1A, Lane 8. 3/dLB, Lane 9. #11



a



b

Fig. 8. Schematic representation of the TAFE patterns.

Pattern 1 was seen in the strains: 972, 442, JKL, UV01, UV02, UV03, UV04, UV05, UV07, UV08 and UV09.
Pattern 2 was seen in the strains: XL1A, 8/dLB (and #11).
Pattern 3 was seen in the strains: DBL, HBL, N1A and THX2A.
Pattern 4 was seen in the strains: 3/dLB and #11.

1
2
3
4

3.3.2. Hybridisation of Chromosome Specific Probes

S. pombe genes that were specific for each chromosome were radiolabelled by random priming. These probes were hybridised to vacuum blotted filters of the TAFE gels in an attempt to determine the order of the chromosomes and the nature of the chromosomal rearrangement.

All of the *S. pombe* genes used hybridised to the *S. malidevorans* strains and were specific for a particular chromosome. In the wild type, chromosome I probes hybridised to the largest chromosome; chromosome II probes hybridised to the middle chromosome and chromosome III probes hybridised to the third chromosome (see Plates 2, 3 and 4). The order of the chromosomes for all strains studied is schematically pictured in Fig.8.

In the mig$^-$ strains, the chromosome II probes hybridised to the altered chromosome. In the case of pattern 4 they hybridised to the top band which was also hybridised to by chromosome I probes. Therefore chromosome II was shown to have a change in mobility which is consistent with an increase in size.

The gel photos and autoradiographs for *cdc 25, cdc 2* and *rDNA* shown (Plates 2,3 and 4) are representative samples of those from each chromosome. The other five probes used (photos not shown) gave the same results as pictured. No cross-hybridisation was detected between the genes specific for chromosomes I, II or III and either of the other chromosomes. The chromosome II probes light up the chromosome III position in some lanes (Plate 3 b,Lane 2). This is most likely due to large degradation products of chromosome II. The fuzzy bands correlate with ethidium bromide stained DNA- smears on the photos (Plate 3 a,Lane 2). All of the probes hybridised to the lowest band, confirming that it was due to degradation products of all three chromosomes.

3.3.3. Separation of *Not* I fragments on CHEF

The plugs of wild type *S. pombe* and *S. malidevorans*, #11 and XL1A were digested with the restriction enzyme *Not* I. The plugs were run on CHEF gels (conditions described in photos, Plate 5 a, b, c) using *Sacc. cerevisiae* and uncut *S. pombe* 972 as size standards.

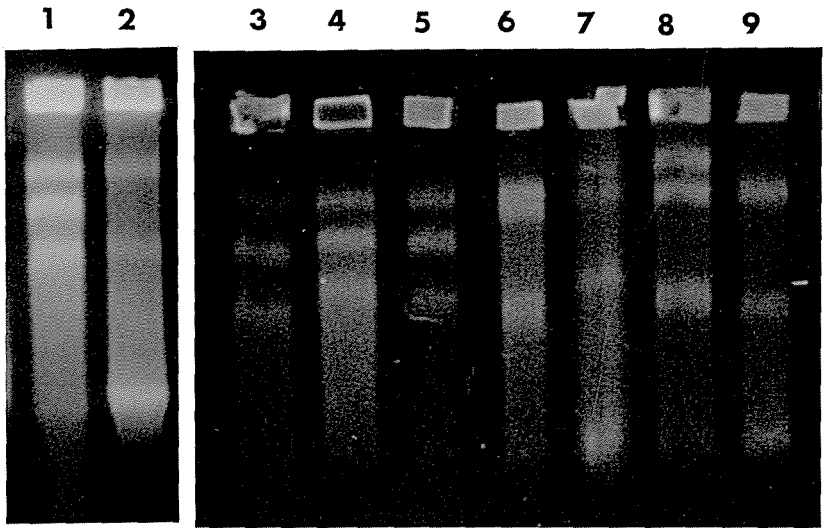
A similar banding pattern was observed for all the cut strains. There were some minor differences (Plate 5) which could be explained by fragments comigrating in one lane but not another (Murray, pers. comm.). The smaller fragments (85-600 kb) were difficult to separate under the conditions used (Plate 5 b), as were the larger fragments (Plate 5 c) which require a shorter switch time to separate further.

The sizes of the *S.pombe* fragments in this study appear slightly different to those reported by Fan *et al* (1989).

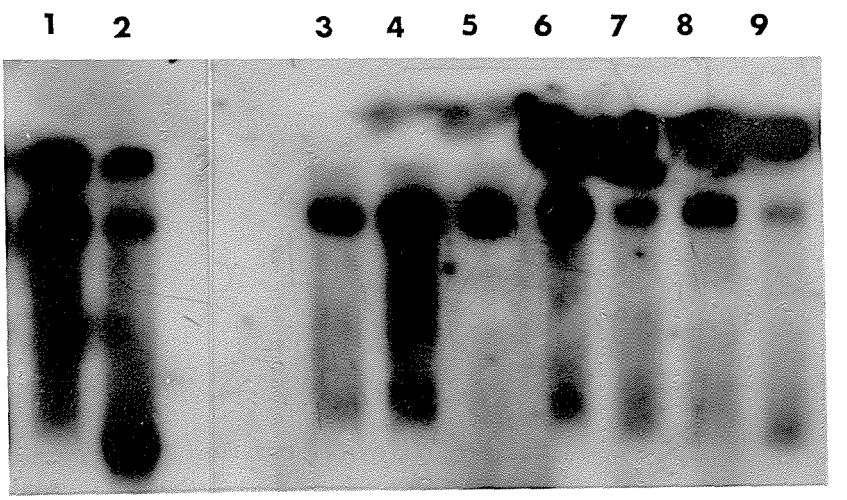
Plate 2. Hybridisation of the chromosome I *cdc 25* gene probe.

a The TAFE gel.
Lane 1. 442, Lane 2. #11, Lane 3. 972, Lane 4. JKL, Lane 5. HBL, Lane 6. 8/dLB, Lane 7. XL1A, Lane 8. #11, Lane 9. 3/dLB

b The autoradiograph.
Lane 1. 442, Lane 2. #11, Lane 3. 972, Lane 4. JKL, Lane 5. HBL, Lane 6. 8/dLB, Lane 7. XL1A, Lane 8. #11, Lane 9. 3/dLB



a

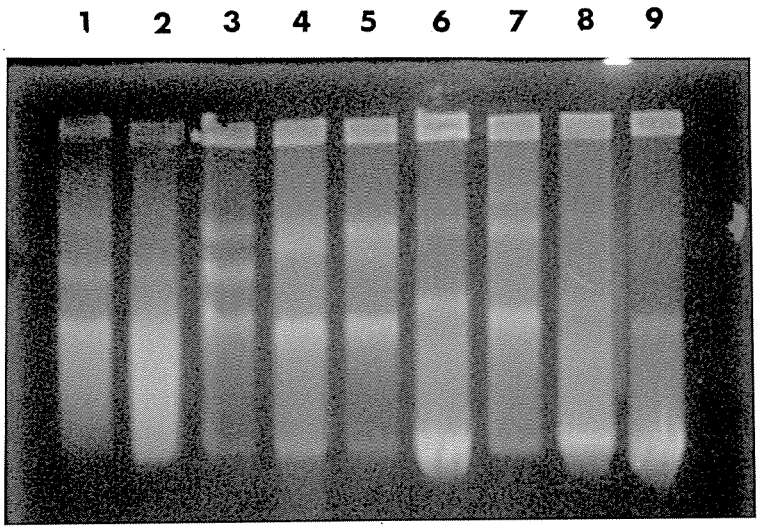


b

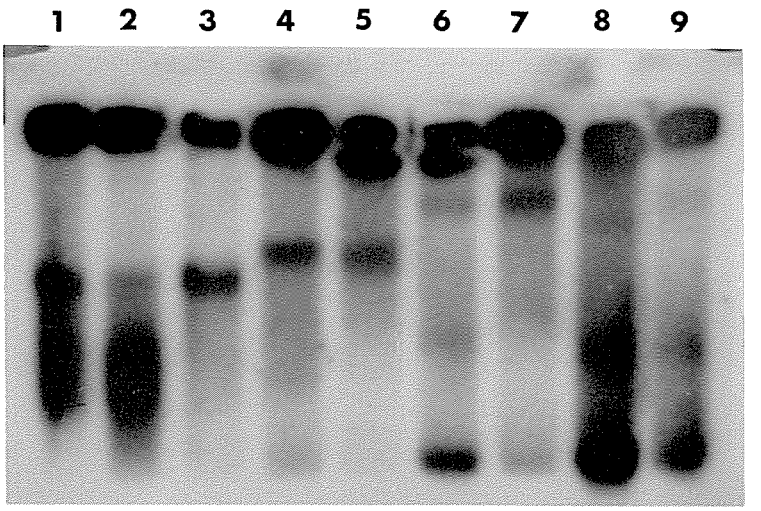
Plate 3. Hybridisation of the chromosome II *cdc 2* gene probe.

a The TAFE gel.
Lane 1. 972, Lane 2. 442, Lane 3. JKL, Lane 4. DBL, Lane 5. HBL, Lane 6. 8/dLB, Lane 7. XL1A, Lane 8. 3/dLB, Lane 9. #11

b The autoradiograph.
Lane 1. 972, Lane 2. 442, Lane 3. JKL, Lane 4. DBL, Lane 5. HBL, Lane 6. 8/dLB, Lane 7. XL1A, Lane 8. 3/dLB, Lane 9. #11



a

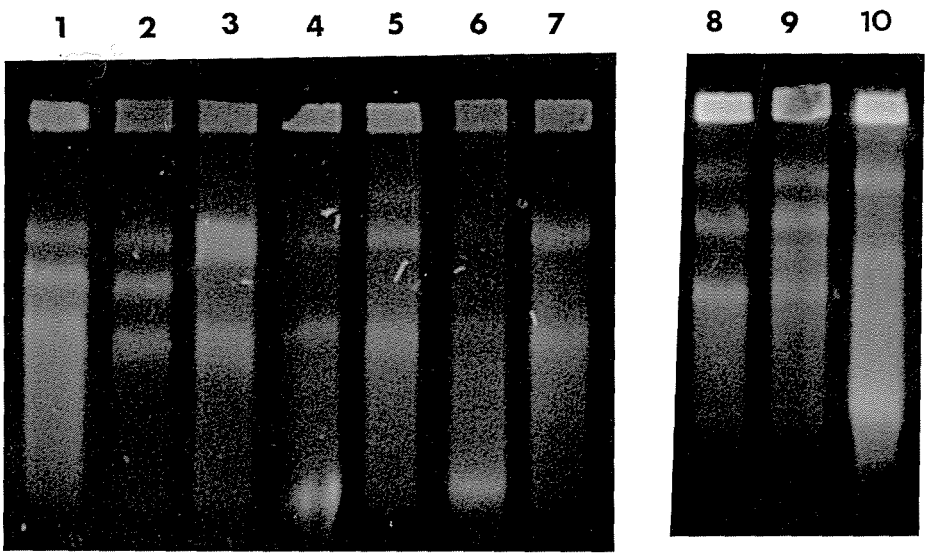


b

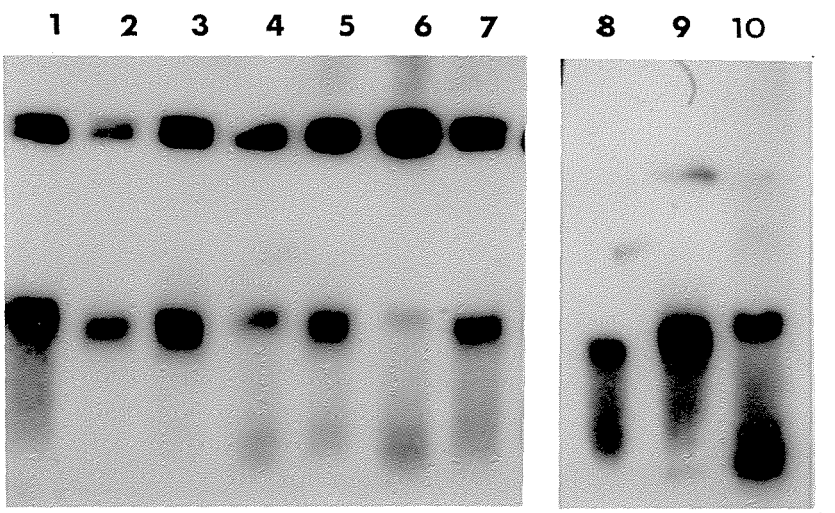
Plate 4. Hybridisation of the chromosome III *rDNA* gene probe.

a The TAFE gel.
Lane 1. 972, Lane 2. 442, Lane 3. JKL, Lane 4. DBL, Lane 5. 8/dLB, Lane 6. XL1A, Lane 7. 972, Lane 8. 442, Lane 9. #11

b The autoradiograph.
Lane 1. 972, Lane 2. 442, Lane 3. JKL, Lane 4. DBL, Lane 5. 8/dLB, Lane 6. XL1A, Lane 7. 972, Lane 8. 442, Lane 9. #11



a



b

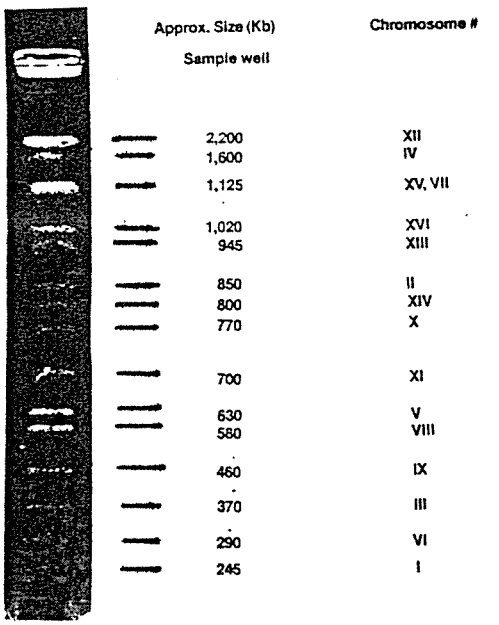
Plate 5. *Not* I fragments on CHEF.

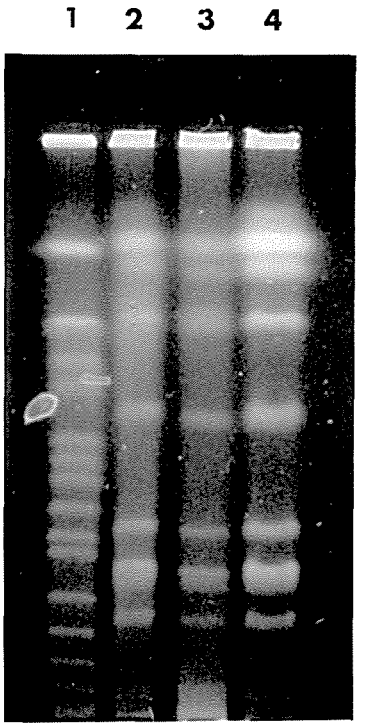
a Comparision of 442, #11 and XL1A.
Electrophoresis was carried out on a 0.6% agarose (Sigma, Type 1) gel at 6 V/cm for 15 hours using a 60 sec switch time then for 8 hours using a 90 sec switch time.
Lane 1. *Sacc. cerevisiae*, Lane 2. *Not* I cut 442, Lane 3. *Not* I cut #11, Lane 4. *Not* I cut XL1A

b Separation of the smaller fragments.
Electrophoresis was carried out on a 0.7% agarose (Sigma, Type 1) gel at 6 V/cm for 15 hours using a 60 sec switch time.
Lane 1. *Sacc. cerevisiae*, Lane 2. *Not* I cut 972, Lane 3. *Not* I cut 442, Lane 4. *Not* I cut #11, Lane 5. uncut 972

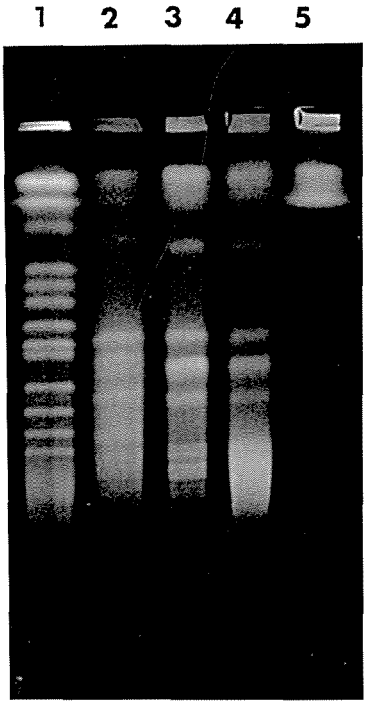
c Separation of the larger fragments.
Electrophoresis was carried out on a 0.6% agarose (BioRad, chromosomal grade) gel at 1.8 V/cm for 15 hours using a 120 sec switch time, then 13 hours with a 450 sec switch and finally for 45 hours with a 27 min switch time.
Lane 1. uncut 972, Lane 2. *Sacc. cerevisiae*, Lane 3. *Not* I cut 972 Lane 4. *Not* I cut 442, Lane 5. *Not* I cut #11, Lane 6. uncut 972

Sizes of the *Sacc. cerevisiae* chromosomes





a



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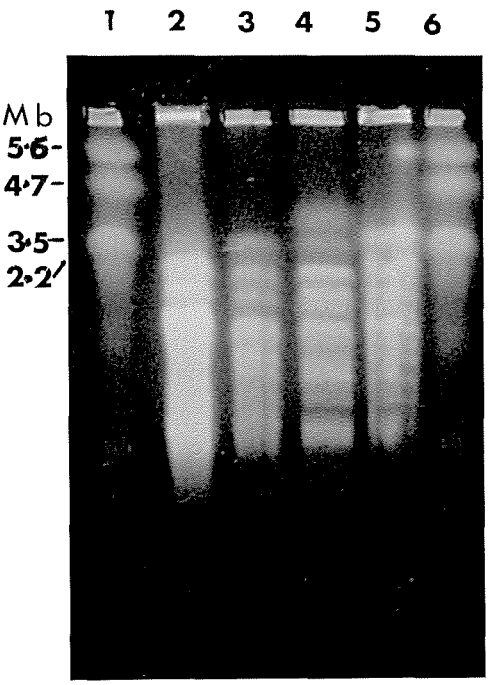

c

4. DISCUSSION

4.1. THE MUTANT CHARACTERISTIC

4.1.1. Enzyme Analysis of fermentation data

Fermentation trials of the five mig$^-$ mutants studied show similar patterns of growth, with respect to cell number and glucose and L-malic acid utilisation (Figs 3-7, Tables 1-5). The #11 pattern is the same as that previously characterised using HPLC (Rodriguez and Thornton, 1988), where glucose is hardly used at all and essentially all of the L-malic acid is used. Once the cells are in stationary phase (approximately 3 x 10^7 cells/ml) they have only used 20% of the glucose and more than 99% of the L-malic acid initially present. This is in contrast to wild type 442 which also uses 99% L-malic acid, but around 80% glucose (Rodriguez and Thornton, 1989).

This implies that these five mutants may have the same mutation or, if they are mutant in different genes, the mutations have similar effects on malate and glucose pathways.

4.2. COMPLEMENTATION TESTS

4.2.1. Auxotrophic Markers

Crosses of pairs of strains were originally carried out by the procedure of Gutz *et al* (1974). Because of difficulties in genetical analysis, such as the bias towards prototrophic haploids and the aberrant segregation ratios, this method was found to be unsuitable. The selection for diploids on MM generally resulted in selection of haploids which were prototrophic for all markers. This is due to mitotic recombination occurring between the mated strains followed by sporulation (Moreno *et al*, 1990). The method of Moreno *et al* (1990) was therefore used. Apart from the large proportion of prototrophs, the mig$^-$ classes were under-represented causing aberrant spore ratios. This is probably due in part to the low spore viability reported by Cambourn-Theewis (1990) and to *S. malidevorans* not being a "lab strain".

The 3/dLB x Spk 3 cross had a 1:1 ratio of mig$^-$:mig$^+$ and of ura$^-$:ura$^+$, therefore it is valid to look for linkage of the mig and ura markers. The number of recombinants was less than the number of parentals suggesting that there is linkage between these markers and, as the recombinant classes are 24% of the total spores, the map distance for these two markers is 24 map units. The *ura* 4 gene, however, is on chromosome III and it is not known how the apparent linkage of *ura* 4 and the mig$^-$ mutation correlates with the observed association of mig$^-$ with an altered chromosome II.

4.2.2. Complementation Tests

The method of Gutz *et al* (1974) was used for complementation analysis. Diploids were selected for on MM + MAL plates to determine that mating had occurred and, because of the problem of prototrophic haploids growing on the MM plates, colonies from these plates were analysed further.

Most of the crosses gave both mig^- and mig^+ colonies on the MM + MAL plates (Table 7). Further analysis of some of the colonies (Table 8) showed that of the mig^+ colonies, some were diploids and many were spore clones. Reversion was discounted as none of the parental strains plated out showed any reversion and the reversion frequency of the mig^- mutation is lower (1×10^{-7}) than the large proportion of mig^+ colonies found in almost every cross. A control cross of 8/dLB with itself was carried out. No complementation was expected in this cross and so all of the diploid colonies should have been mig^-. All of the diploids found were mig^+. No reversion of the strain was seen before mating and it is unlikely that all of the mating cells reverted in the cross to form mig^+ diploids. Therefore, there appears to be some factor, other than complementation or reversion, occurring during mating to give mig^+ colonies.

The diploids (Table 7) were sporulated and the spores analysed by random spore analysis to determine the true diploids from any haploids which had grown up (Table 8). There was evidence of diploids from colonies where several spore classes were found (eg the 8/dLB x 3/dLB cross), and also of haploids from colonies whose spores lacked segregation (eg the mig^+ "diploid" of the 3/T x 8/dLB cross). Nine mig^+ and 6 mig^- colonies from Table 7 appeared to be diploids and 14 mig^+ and 8 mig^- colonies were haploid. The mig^+ colonies tested from the crosses 3/dT x DBL and 8/dLB x #11 gave one major class ($m^+a^+l^+$) and one or two minor classes of spores. It is likely that the mig colonies were haploid, with the minor spore classes being of uncertain significance. However, the possibility that the mig colonies were diploid cannot be excluded. Also a mig^- colony from the #11 x TVA cross gave 47 $m^+a^+l^+$ spores and 5 $m^-a^+l^+$ spores. It is unknown how a mig^- colony could give mostly mig^+ spore clones. In the #11 x TVA cross it is unlikely that a cell reverted, grew up and then sporulated giving mig^+ spores, as the colonies sporulated were taken from MM + MAL plates and had been shown to be mig^- by replica plating. Any reversion that occurred would have to have occurred during sporulation, which is unlikely in such high numbers. 47 of the 52 spores analysed have apparently lost the mig^- phenotype during the process of sporulation and germination, by some mechanism other than reversion.

In the original complementation tests, haploids that were mig^- colonies could come from either complementing or noncomplementing strains but haploids which were

mig$^+$ colonies were not necessarily from complementing strains because of the apparent occurrence of an additional factor that affects the mig phenotype. Most of the complementation crosses have one or both classes of haploids so complementation groups cannot be assigned on the basis of the haploid colonies. The diploid colonies, however, do give an indication of complementation, where a mig$^+$ diploid implies complementation and a mig$^-$ diploid suggests noncomplementation. Most of the crosses include either a mig$^+$ or a mig$^-$ diploid. The exceptions are the crosses involving JKL, where both mig$^+$ and mig$^-$ diploids may be found within a single cross between two mutants. It may be significant that JKL is the only mig$^-$ mutant with a wild type TAFE pattern.

The mig$^+$ diploid colonies from the crosses 8/dLB x 3/dLB and #11 x DBL were sporulated, the mig$^-$ spore mated with the parent strains and these crosses tested for their mig phenotype (Table 9). The spore clones gave the complementation patterns expected if the parents were mutant in different genes, although there was an extra class present where the spore clones complemented with both parents. It is difficult to see how it is possible to get mig$^+$ colonies (and therefore complementation) with both parents when the spore clones have mig$^-$ mutations that must be derived from one of the parents. This strongly suggests the interference of some other factor, as does the apparent complementation with both parents of two mig$^-$ spores from a mig$^-$ haploid (#11 x DBL).

The mig$^-$ spore clones from a mig$^+$ diploid of the #11 x JKL cross, were mated with the parents. Eighteen of the 20 spore clones tested complemented with both parents, which is not expected from complementing strains, while two spore clones complemented with #11 only. The ability of the spore clones to give mig$^+$ diploids with both parents means that no conclusions can be reached on whether the two parents are in the same complementation group.

Further analysis of a selection of colonies from the complementation tests has allowed the resolution of several of these colonies as demonstrating complementation or noncomplementation. The data indicates that the complementation tests can be scored on the basis of the mig phenotypes of the diploid colonies. The crosses with JKL, however, can only be characterised by further analysis of the mig$^-$ spore clones. The complementation results are summarised in Table 10 and indicate the presence of at least three complementation groups for the mig$^-$ phenotype. DBL, 8/dLB and 3/dT appear to be in one group; #11 and HBL in another; and 3/dLB in a third. TVA is in a different complementation group to #11 and HBL. This confirms the preliminary work reported by Parker (1988) and by Rodriguez (pers. comm.). The analysis shows the necessity of being able to identify diploid colonies on MM before testing

Table 10. Summary of complementation results.

A summary of the complementation groups based on the results from Tables 7, 8 and 9.
C. G. = complementation group, "-" = no complementation, "+" = complementation.

	?	C. G. 3	C. G. 2		C. G. 1		
Strains	TVA	3/dLB	#11	HBL	DBL	8/dLB	3/dT
3/dT						-	
8/dLB		+			-		
DBL			+				
HBL	+	+	-				
#11							
3/dLB							
TVA							

for complementation of the mig phenotype. Further characterisation of the mig$^-$ spore clones from selected crosses is also important to confirm the complementation results.

The three genes may be in the same metabolic pathway affecting glucose and malate metabolism, possibly the glycolytic pathway. #11 has been shown to have unaltered glucose and malate transport systems (Cambourn-Theewis, 1990). It is unknown if this is also found in the other two complementation groups. Further analysis of the mutants to complete the complementation analysis will allow the selection of suitable mutants for further characterisation of the molecular effects.

4.3. CHROMOSOMAL WORK

Karyotyping of *S. pombe* by various methods has lead to the discovery of three chromosomes in the haploid. Methods such as light microscopy and mitotic staining (Robinow, 1977), genetic analysis (Gygax and Thuriaux, 1984) and electrophoretic karyotyping (Fan *et al*, 1988) have been used.

Murray (1989) looked at the electrophoretic mobility of *S. malidevorans* #11. She reported that #11 has an altered pattern on TAFE with respect to the wild type, where chromosome II appeared larger than chromosome I. Further work with DNA content assays and UV survival curves, showed that *S. malidevorans* 442, like *S. pombe*, was haploid. #11 however, was neither haploid nor diploid but did contain extra DNA (Murray, 1989).

Research on *Sacc. cerevisiae* centromeres showed that regions of the centromere have hotspots for UV damage, also UV induced damage in the centromere can cause translocations through recombination (Resnick *et al*, 1987). Recombinational hotspots have been found in the *S. pombe ade6-M26* mutation (Ponticelli *et al*, 1988; Schuchert and Kohli, 1988). It is possible that *S. malidevorans* may have a hotspot on chromosome II which, when subjected to UV, causes some sort of chromosomal rearrangement resulting in the mig$^-$ phenotype.

4.3.1. Chromosomal Banding Patterns on TAFE

The chromosomal banding patterns of wild type *S. malidevorans* and *S. pombe* are similar, supporting the inclusion of *S. malidevorans* in the same species as *S. pombe*. All mig$^+$ mutants have the wild type pattern, however an altered pattern involving chromosome II is seen in all mig$^-$ mutants except for one. The mig$^-$ mutants show not only a change in chromosome II, but also differences within this change (Fig 8.). This implies that there are several different alterations in chromosome II that can result in the mig$^-$ phenotype. Variability was also noticed within the same strain (eg #11, DBL and HBL). This variability may reflect an

artifact of the chromosome preparations or the TAFE running conditions, rather than an actual chromosome change. It does appear however, that only chromosome II is involved. The change in chromosome II could be caused by a rearrangement, such as a duplication, or a change in conformation, such as a ring chromosome.

A possible explanation for the variability within a strain (eg in DBL and HBL) is that the initial mig$^-$ mutation is unstable. The chromosome patterns initially still appear wild type but chromosome II is unstable. Then, over a period of extended growth of the mutant, chromosome II changes to give a final constant, altered pattern. Further study is necessary to investigate these observations. This does not explain why the strain JKL did not change like DBL and HBL after several months of isolation, neither does it explain the variability seen within the same preparation of #11 plugs. The latter might be a result of a change in conformation of chromosome II because of lost proteins or breaks caused from long storage. The initial JKL mig$^-$ mutation either does not involve a chromosomal change, or involves one that is stable and does not result in a detectable change in mobility.

Variations in *S. malidevorans* chromosome III mobility were noticed. Chromosome III has either of two positions, in line with *S. pombe* chromosome III or with a slightly decreased mobility. Either position was considered normal since it was seen in most *S. malidevorans* strains, both mig$^-$ and mig$^+$. This was possibly due to variations in the amount of chromosomally-bound protein affecting chromosome conformation and therefore mobility. As chromosome III is significantly smaller than the other two chromosomes, the presence/absence of protein could make a noticeable difference to mobility.

The lowest band in the photographs was shown to be degradation products of the three chromosomes and not a fourth chromosome as it gave a signal with all of the probes used.

The fact that so many mutants were easily isolated and have "abnormal" chromosome patterns, while mig$^+$ auxotrophic mutants have wild type patterns, suggests that chromosome II is somehow involved in the mig$^-$ phenotype. It is possible that the mating-type locus is involved. This is supported by two observations: the mating type locus is found on chromosome II, which is altered in all but one mig$^-$ mutant; and all of the mig$^-$ mutants are homothallic. To date no mig$^-$ mutants have been isolated from heterothallic strains and no heterothallic mig$^-$ spore clones have been detected in crosses with heterothallic strains (Cambourn-Theewis, 1990). This suggests that the mating type locus has an important role in the mig$^-$ mutation.

Switching of the mating-type in fission yeast occurs at the mating-type locus (MTL). The MTL consists of three closely linked copies of mating-type information

on chromosome II. The three loci are *mat 1*, *mat 2* -P and *mat 3* -M, with the *mat 1* locus determining the cells' mating type. Switching of the mating type is initiated by a double-stranded DNA break at the MTL. It takes two generations to produce a switched cell and not every cell switches (Nielsen and Egle, 1989).

In *S. pombe* the frequency of mating type switching is decreased and UV sensitivity increased, by mutations in the switching genes. Mutations in these genes can also effect recombination (Schmidt *et al*, 1989). Further investigations might indicate whether a UV-induced error in mating type switching plays a role in the mig$^-$ mutation.

4.3.2. Hybridisation of Chromosome Specific Probes

Nine unique *S. pombe* genes which were specific for a particular chromosome were used for Southern hybridisation (Fig. 2). The results confirmed the identity of the bands and that chromosome II was the altered chromosome. Any cross-hybridisation to other chromosomes would be because a translocation had occurred within the yeast genome. No cross-hybridisation was seen between any of chromosome I or III probes and chromosome II. As the probes used were located near either ends of each chromosome, it is unlikely that chromosomes I and III are involved in the altered chromosome. However the possibility of the involvement of regions of chromosomes I and III, for which probes were not available, cannot be excluded. The results presented in this study imply that only chromosome II is involved and that it has a slower mobility than the wild type. This is most likely due to either a ring chromosome II or a duplication of all or part of chromosome II.

Chromosome duplications cannot be detected directly on autoradiographs with a single probe by comparison of signals between adjacent lanes. This is because it is very difficult to control the amount of DNA in different chromosome preparations and the signals obtained on an autoradiograph are not always comparable to the amount of DNA present. The chromosomes could be examined for the possible duplication of chromosome II using a mixed probe. The mixed probe would consist of a chromosome II probe and another chromosome (eg. chromosome III). As chromosome III is not duplicated its signal would act as an internal standard for each lane. If there is no duplication of chromosome II the intensities of chromosomes II and III would be expected to be the same, if a duplication had occurred the intensity of II should be twice that of chromosome III.

4.3.3. Separation of *Not* I fragments on CHEF

Not I is a restriction enzyme suitable for chromosome analysis. As the recognition sequence is eight base pairs long, cleavage of the DNA with *Not* I gives a relatively

small number of fragments. As shown by Fan *et al* (1988), *S. pombe* has 14 detectable *Not* I sites: 9 sites on chromosome I, 5 sites on chromosome II, and no sites on chromosome III. The fragments range in size from 4.5 kb-3.5 Mb. If the altered chromosome II was due to a ring chromosome, *Not* I digestion should have yielded an extra, large, fragment and the loss of the two telomere fragments (Rochet and Fan, 1990). Alternatively, if the variation was due to a duplication or translocation, there would also be an extra band(s) and a loss of another band although not necessarily a telomere fragment.

The results shown of *Not* I complete digests of 972, 442, #11, and XL1A show no significant differences. The conditions used on CHEF in this study, however, were not appropriate for complete resolution of all bands. The smaller fragments in the 85-600 kb range proved difficult to separate. The conditions for the larger fragments (1.2-3.5 Mb) require a shorter switching time to allow greater separation of the bands.

Further study needs to be carried out with *Not* I digests before conclusive results are drawn. Better results would be obtained if the three chromosomes could be separated prior to *Not* I digestion as this would remove the problem of separation of the similar sized fragments from different chromosomes.

5. CONCLUSION

Four mig$^-$ mutants were isolated and characterised along with four previously isolated mig$^-$ mutants. The mutants have the same phenotypes and similar glucose and malate utilisation, suggesting that the mig$^-$ mutation(s) involve the same metabolic pathway. The differences between them lie in their chromosomal patterns, as determined on TAFE, and their complementation groups. Complementation tests suggest that there are three genes involved in the malate-dependent mutation.

The odd complementation patterns, where two mig$^-$ strains mate to give a mig$^+$ colony, were not expected as evidence from genetical analysis implies that there has been no complementation between the two strains. This suggests that there is some other factor resulting in the resolution of the mig$^-$ mutation to a mig$^+$ phenotype. This factor occurs independently of complementation, is seen in most of the matings at diploidisation and meiosis and to some extent, masks the complementation results. It is unlikely that this change to mig$^+$ is due to reversion, as the frequency of reversion is too low to give the large number of mig$^+$ colonies found in every cross. This may be a consequence of the chromosomal rearrangements shown on TAFE. A possible explanation for this factor is that there is some interaction between the chromosomes in the mated diploid which can reverse the effect of the chromosome rearrangement in the mig$^-$ mutation. This interaction could involve a recombinational event.

Chromosome II of the mig$^-$ mutants has a slower mobility on TAFE to that of the mig$^+$ strains. This alteration of chromosome II varies between the mig$^-$ mutants and chromosomes I and III do not appear to be involved in this rearrangement. This slower mobility can be explained by an increase in size, due to a duplication of all or part of chromosome II, or a change in conformation, such as the formation of a ring chromosome. If the nature of the rearrangement was either a ring chromosome or a duplication, it would explain the aberrant spore ratios and reported low spore viability. However, a ring chromosome would not explain the variation of the chromosome II mobilities seen in the mig$^-$ strains.

A duplication of part of the chromosome seems the most likely answer. Different amounts of chromosome II could be duplicated to give the varying pattern found, in response to UV hotspots along chromosome II. The duplication could involve the MTL as the mating type of the parental strain reflects the ability to isolate the mutation.

The mig$^-$ phenotype appears to be the result of a rearrangement of chromosome II, with this altered organisation affecting the malate and glucose metabolism (with respect to the wild type). The low spore viabilities, altered spore clone ratios and the apparent ability of non-complementing mig$^-$ mutants to form mig$^+$ diploids or spore clones is also a likely consequence of the rearranged chromosome.

Further study of the mig$^-$ phenotype may increase understanding of glucose and malate metabolism and of the effects of chromosome organisation on patterns of gene expression.

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I didn't do it
Nobody saw me do it
There is no proof

Bart Simpson