Investigations on Malic Acid Utilisation in *Schizosaccharomyces* species

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Abstract

The aims of this investigation were: to determine whether alterations in uptake, or metabolism, of glucose and malate was the cause of malate dependence; to determine the number of genes involved in malate dependence; and to clone the gene(s) involved. A malate dependent mutant, i.e. a mutant that requires both malate and glucose for growth, (mutant 11) of *Schizosaccharomyces malidevorans* 442 was characterised. Malic enzyme activity was increased almost ten-fold. The V_{max} of malate uptake was increased four-fold compared to *S. malidevorans* 442. Uptake of glucose was significantly lower in mutant 11 than the wild-type. The kinetics of glucose uptake by *S. malidevorans* 442 and mutant 11 suggested the presence of two glucose transporters, a high affinity and a low affinity transporter. Only the low affinity transport was apparently altered in mutant 11 compared to the wild-type.

Genetic analysis indicated that the malate dependent mutation is recessive and is the result of a single mutational event. Crosses involving derivatives of mutant 11 and *Schizosaccharomyces pombe* strains did not yield the expected segregation of markers. Tetrad analysis showed that the spore viability was very low. It was not possible, therefore, to determine linkage of the malate dependence locus and any other loci.

All malate dependent strains were apparently homothallic although linkage between the mating-type locus and malate dependence could not be established. The isolation of similar mutants from homothallic strains of *S. pombe*, but not from heterothallic strains,

provided strong support for the requirement of homothallism for malate dependence. The pulse field gel electophoresis karyotypes of mutant 11 and derivatives of mutant 11 suggested the presence of a large chromosomal rearrangement of chromosome 2 that co-segregated with malate dependence.

Malate dependent mutants were not obtained from homothallic *Saccharomyces cerevisiae* MD26.

A malate dependent mutant (WT 6) was isolated from *S. pombe* WT 4 and found to have characteristics similar but not identical to those of mutant 11. WT 6 demonstrated increased utilisation of malate and decreased utilisation of glucose. Malic enzyme activity was not altered in WT 6 compared to the wild-type. Malate uptake was not affected. The karyotype of WT 6 suggested that a chromosomal rearrangement had occurred, but it is not identical to the rearrangement in mutant 11.

The differences in the characteristics of mutant 11 and WT 6 suggested the mutations in these mutants may not be identical. The finding that mutant 11 and WT 6 belong to different complementation groups could explain these differences.

Although differences were found in the uptake of malate and glucose, the inability of malate dependent mutants to grow on glucose implicates a defect in glucose metabolism.

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For Hugh, Micah and Bowser

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Introduction

1. General description and taxonomy of the genus *Schizosaccharomyces*

The genus *Schizosaccharomyces Lindner* comprises four species, *Schizosaccharomyces octosporus, Schizosaccharomyces japonicus, Schizosaccharomyces pombe* and *Schizosaccharomyces malidevorans,* according to taxonomic texts (Barnett *et al.* 1983, Yarrow 1984). The genus is characterised by globose to cylindrical cells, reproduction by fission and production of two to eight spores per ascus.

Schizosaccharomyces pombe was first isolated by Lindner (1893), from an African millet beer called pombe, and is represented by a large number of strains found mainly in fruit juices, molasses, arak mash, Bantu beer and on sugar cane.

S. malidevorans was first described by Rankine and Fornachon (1964). It is represented by just two strains, isolated from grapes. *S. malidevorans* differs from *S. pombe* in its ability to completely decompose malic acid in grape juice or synthetic media, its inability to ferment maltose, production of hydrogen sulphide and the rough appearance of its ascospores (Rankine and Fornachon 1964).

It has been suggested recently that *S. malidevorans* is a variety of *S. pombe* due to the lack of distinguishing characteristics

(Bridge and May 1984), interfertility between the two species (Sipiczki *et al.* 1982), supported by DNA-DNA hybridisation values of 98% between the two species (Sipiczki 1989). The synonomy of the two species is not, however, accepted by all workers (Johannsen 1981).

The 5S-rRNA of the genus *Schizosaccharomyces* has been compared to that of other organisms in order to establish its phylogenetic relationships. There were more differences between *S. pombe* and *Saccharomyces cerevisiae* 5S rRNA than between *S. pombe* and the 5S rRNA of rats or humans (Erdman *et al.* 1983; Komiya *et al.* 1981). The genus *Schizosaccharomyces* is much more closely related to *Neurospora* and *Aspergillus* than to *Sacch. cerevisiae* (Sipiczki 1989). This is interesting because *Sacch. cerevisiae* is often used as a model for lower eucaryotes. *S. pombe's* closer ties with higher eucaryotes was illustrated further by the discovery that *S. pombe* could more accurately splice the introns from the genes of higher eucaryotes than could *Sacch. cereviseae* (Kaufer *et al.* 1985), making *S. pombe* attractive for cloning and expression of eucaryotic genes.

S. pombe has been reported to have three chromosomes on the basis of light microscopy (Robinow 1977), genetic analysis (Gygax and Thuriaux 1984) and by electrophoretic karyotyping (Smith *et al.* 1987, Fan *et al.* 1988). Work done by Murray (1989) using the TAFE karyotyping system showed that *S. malidevorans* 442 has three chromosomes of approximately the same size as *S. pombe* WT 4. Radioactive probing of TAFE gels with a single gene of each of the three *S. pombe* chromosomes has indicated that chromosomes 1, 2, and 3 of *S. pombe* are very similar to those of *S. malidevorans* (Hansen, personal communication).

2. The life cycle of *Schizosaccharomyces pombe* and *Schizosaccharomyces malidevorans*

Both *S. malidevorans and S. pombe* are predominantly haploid yeasts that grow vegetatively by cell fission. Under starvation conditions the sexual cycle is initiated by conjugation of cells. In heterothallic strains of *S. pombe*, mating occurs between h^+ and h^- cells. Other strains are homothallic (h^{90}) and mate in pure cultures because of their ability to switch mating-type. The zygote usually proceeds to meiosis resulting in the formation of asci containing four spores. On rare occasions a zygote may develop into a diploid vegetative cell which will undergo meiosis when it enters stationary phase, forming an azygotic ascus with four haploid spores. Azygotic asci are oval rather than the horseshoe shape of zygotic asci.

The ability of *S. pombe* to switch mating-type in homothallic strains depends on three mating type loci, all linked within 1 centimorgan on the long arm of chromosome 2. One of the loci is active (*mat1*) and there are two dormant loci *mat2-P*, coding for for h^+ information , and *mat3-M*, coding for h^- information (Beach *et al.* 1982b, Beach 1983). Mating-type switching occurs when one of the two dormant loci is transposed into the *mat 1* locus, becoming active (Beach 1983). This switching occurs once every few cell divisions in homothallic strains (Egel 1977, Miyata and Miyata 1981). It is thought that a sequence flanking both silent cassettes but missing from *mat 1,* called H3, may play a role in 'position effect' regulation (Kelly *et al.* 1988). A very similar mating-type system has been found in *S. malidevorans* (Sipiczki *et al.* 1985).

3. Glucose and malate metabolism in yeast

Species of both *Schizosaccharomyces* and *Saccharomyces* utilize sugars for growth and energy. D-glucose, which is the commonest source of energy, is catabolised respiratively to carbon dioxide and water, or fermentatively to carbon dioxide and ethanol.

The first step in metabolism of glucose is its uptake. *Sacch. cerevisiae* has two glucose transporters, a high affinity glucose transport (K_m 2 mM), that is induced at low concentrations of glucose and a low affinity transport (K_m 20 mM) (Does and Bisson 1989). The activity of high affinity transport in *Sacch. cerevisiae* is dependent on hexokinase activity (Lang and Cirillo 1987, Bisson 1988). Although the low affinity transport is constitutive, its activity is inversely proportional to the activity of the high affinity system. This observation suggests that there is some regulatory relationship between the two processes (Ramos *et al.* 1988). Two glucose transporters are also found in several other yeast species (Does and Bisson 1989, Barnett and Sims 1976a, Barnett and Sims 1976b), however no similar work has been done in *S. pombe.*

Both fermentation and respiration of glucose depend on a functional glycolytic pathway. Identical steps in glycolysis have been demonstrated in *Saccharomyces* species (Maitra and Lobo 1971; Lam and Marmur 1977) and *S. pombe* (McDonald and Tsai 1989). Under aerobic conditions, the pyruvate formed by glycolysis can be converted to acetyl-CoA and enter the TCA cycle. The TCA cycle yields much more energy than alcoholic fermentation, during which pyruvate is converted to acetaldehyde and then ethanol. Alcoholic fermentation occurs in the absence of oxygen, or when glucose, and therefore energy, is in excess (Fiechter *et al.* 1981).

S. pombe and *S. malidevorans* grow on a very limited number of substrates, other than glucose, as sole carbon source (Barnett *et al.* 1983, Yarrow 1984). *S. pombe* and *S. malidevorans* are K- yeasts, i.e. they cannot grow on malate as sole carbon and energy source. Glucose is required in order to utilise externally provided tricarboxylic acid cycle intermediates. Barnett and Kornberg (1960) studied the enzyme systems involved in the tricarboxylic acid (TCA) cycle intermediates of K+ yeasts, i.e. those yeasts that can use malate as a sole carbon and energy source, and K- yeasts and concluded that the inability to grow on these substrates without glucose is due to a "permeability barrier" rather than lack of any major metabolic pathway. The implication is that glucose is required for the transport of TCA cycle intermediates.

Malate is actively transported in *S. pombe* by a common dicarboxylic acid transport system (Osothsilp and Subden 1986a). The yeasts *Kluyveromyces lactis* and *Candida sphaerica,* and the fungus *Neurospora crassa,* also have common dicarboxylic acid transporters (Zmijewski and MacQuillan 1975, Corte-Real *et al.*

1989, Wolfinbarger and Kay 1973). Common dicarboxylic acid transport systems are also found in several bacteria, including *Rhizobium leguminosarum* (Ronson *et al.* 1981) and *Rhizobium meliloti* (Hornez *et al.* 1989).

Rodriguez and Thornton (1990) report that malate uptake by *S. malidevorans* is reduced at low concentrations of glucose (28 mM). They suggest that this reduction is due to a reduced amount of energy for the active transport of malate. An alternative explanation for the glucose requirement may be that it is needed to induce the malate uptake system. Baranowski and Radler (1984) found that the malate transport system in the fructophilic yeast *Zygosaccharomyces bailii* was induced in glucose grown, but not in fructose grown, cells. Malate transport was induced by glucose and also inhibited by glucose in this organism (Herzberger and Radler 1988). *K. lactis* also requires glucose for the induction of a dicarboxylic acid transport system (Corte-Real *et al.* 1989). Malate transport by *S. pombe* was reported to be constitutive because it was present whether the cells were grown with or without malate (Osothsilp and Subden 1986a). This finding does not rule out the possibility that glucose induces malate uptake.

Once inside the cell, malate can be utilised by different pathways, depending on the culture environment. Malate can enter the TCA cycle directly, or be converted to pyruvate by malic enzyme to be used either in the TCA cycle, used in gluconeogenesis, or be converted to ethanol.

4. Malate utilisation in yeast and the deacidification of wine

Malic acid and tartaric acid are the main acids present in grape must. The decomposition of malic acid is important in the deacidification of wine. Malolactic fermentation by bacteria is the usual biological method of deacidification, however, alternatives to this slow process are desirable (Snow 1985). There is, therefore, considerable interest in understanding the utilisation of malate by yeast.

Sacch. cerevisiae strains are unable to completely utilise the malate in grape must, with 3-45% of the malate being decomposed (Rankine 1966). Malic enzyme is the main enzyme involved in this process in both aerobic and anaerobic growth (Mayer and Temperli 1963, Fuck and Radler 1972, Osothsilp and Subden 1986b). The low utilisation of malate is apparently due to the low malic enzyme activity of *Sacch. cerevisiae* (Kuczynski and Radler 1982) and the lack of active transport or facilitated diffusion of malate by *Sacch. cerevisiae* (Baranowski and Radler 1984).

S. pombe strains are able to use as much as 98% of the malate in grape must (Snow and Gallander 1979), while *S. malidevorans* is unique in its ability to utilise all of the malate in grape must (Rankine and Fornachon 1964, Rankine 1966). The malic enzyme activity of *S. pombe* is fifteen times that of *Sacch. cerevisiae* (Kuczynski and Radler 1982). This, and the finding that malate is actively transported (Osothsilp and Subden 1986a), probably accounts for the difference in malate utilisation by *S. pombe* compared with *Sacch. cerevisiae*.

Although *Schizosaccharomyces* species have the ability to deacidify grape must, they produce undesirable flavours and excessive hydrogen sulphide, precluding commercial use of this yeast for deacidification (Rankine 1966, Gallander 1977, Snow and Gallander 1979). Rankine (1966) obtained mutants of *S. malidevorans* that did not produce hydrogen sulphide; however, these mutants reverted on subculturing. A mutant of *S. malidevorans* that has properties ideal for deacidification of wine was obtained by Rodriguez and Thornton (1989). This strain was designated mutant 11. This organism used all the malate in grape juice without producing hydrogen sulphide and without using much of the glucose in the juice. After depletion of malate from the juice, mutant 11 stops growing (Rodriguez and Thornton 1988).

Attempts have been made to clone the gene responsible for malolactic fermentation from *Lactobaccillus delbruekii* into *Sacch. cerevisiae*, but high expression of the bacterial gene in yeast was not achieved (Williams *et al.* 1984). If a gene for malate utilisation were cloned from another yeast into *Sacch. cerevisiae*, fewer problems with expression would be expected.

5. Mutant 11

Mutant 11 and other similar mutants were isolated from *S. malidevorans* 442 following UV mutagenesis and selection on indicator plates containing glucose, malate and a pH indicator, bromocresol green (Rodriguez and Thornton 1989). These mutants were light blue on the indicator plate compared with the blue/green parental strain (Plates 1 and 2). The mutants were strictly malate dependent, i.e. required both malate and glucose for growth. Pyruvate, succinate and fumarate failed to alleviate malate dependence. They were unable to sporulate and did not form asci readily when mated with other *Schizosaccharomyces* strains (Lubbers 1987, Rodriguez, personal communication). Fermentation trials in minimal media containing 0.3% malate, 10% glucose and a nitrogen source showed that mutant 11 was capable of using malate faster than the wild-type, while using only 20% of the glucose in the medium (Rodriguez and Thornton 1989). Mutant 11 did not reach the cell numbers at stationary phase that the wild-type did. Whereas the wild-type viable cell numbers declined rapidly after reaching stationary phase, the mutant cells did not die. As far as the authors are aware no pleiotrophic mutants such as these have previously been described (Rodriguez and Thornton 1989).

Possible explanations for the lack of previous reports of these mutants were firstly, that mutants were usually selected for on complete medium, that does not contain malate. Since these mutants required malate they would not be isolated by such a method. Secondly, it was thought that *S. malidevorans* may differ sufficiently from other yeast species to make the isolation of novel mutations possible (Rodriguez and Thornton 1989). This was supported by the inability of one investigator to isolate similar mutants from the very closely related species *S. pombe* (Lubbers 1987).

6. Aim of this investigation

The objectives of this investigation were:

1. To determine whether the mutation(s) that results in malate dependence is dominant or recessive and whether the phenotype is the result of one or more mutations. The answers to these questions will affect the strategy used for cloning the gene or genes involved in the mutation(s). If the mutation is recessive a wild-type library is appropriate. If the mutation(s) is dominant a mutant library should be made.

2. To investigate the physiology and metabolism of the wild-type and malate dependent mutants in order to identify the gene(s) affected by the mutation. The malate dependence of these mutants suggests the involvement of uptake or utilisation of malate. The metabolism of tricarboxylic acids by yeast, especially in *S. pombe*, has been not been widely investigated. Whether malate is metabolised by *S. malidevorans* in the same way as *S. pombe* is unknown.

3. To investigate the reason for the failure to obtain malate dependent mutants in a very closely related strain, *S. pombe*. If malate dependent mutants can only be

isolated from *S. malidevorans*, this would be a significant difference between the two species.

4. To clone the gene(s) altered in mutant 11, with a view to the eventual transformation of *Sacch. cerevisiae* into a strain that can completely deacidify wine.

Materials and Methods

1. Microbial strains and maintenance

The yeast and bacterial strains used in this study are listed in Tables 1 and 2. Yeast strains were maintained on YMM agar slopes at 4^{o}C and were subcultured at intervals of 6-12 months. Bacterial strains were stored at -70^{o}C in Luria Broth containing 15% glycerol. Bacterial strains were grown at 37^{o}C unless otherwise stated. Yeast strains were grown at 30^{o}C unless otherwise stated.

2. Media

2.1. YM and YMM media

YM medium (Difco Laboratories) is a complete yeast medium. All media was made up using distilled deionised water. YMM medium is YM with 3 g/l malic acid (Sigma Chemical Co., HPLC grade). The pH of YMM was adjusted to 4.5 with 3 M KOH (YMM). Solid media contained 20 g/l agar (Difco Laboratories). Media were sterilized for 20 minutes at 110^{o}C, to prevent caramelisation of sugar.

2.2. Yeast minimal media (MM, MMM)

Yeast minimal medium (MM) contained 6.7 g/l yeast nitrogen base without amino acids (Difco Laboratories) and 20 g/l glucose (BRL). For the solid medium, 20 g/l agar (Difco) was added. MMM medium is MM with 3 g/l malic acid. The pH of MMM was adjusted to 4.5 with 3 M KOH before the addition of agar. MM and MMM plates with reduced glucose were made with 0.5 g/l and 1 g/l glucose. When plates with a pH less than 4.5 were required, the agar was autoclaved separately in half the volume of deionised water. Minimal media were supplemented by the addition of

Table 1: *Schizosaccharomyces* and *Saccharomyces* strains used in this study.

STRAIN	GENOTYPE/PHENOTYPE	SOURCE (or method of construction)
Schizosaccharomyces malidevorans **strains**		
S. malidevorans 442	wild-type spo$^+$	AWRI 158
Mutant 11	mig$^-$ spo$^-$	S Rodriguez
WT11	*ade* spo$^+$	UV from *S.malidevorans* 442
WT12	*ade* spo$^+$	UV from *S.malidevorans* 442
Schizosaccharomyces pombe **strains**		
WT1 h$^+$	*ura 4*-D18	J Kohli
WT2 h$^-$	*ura 4* -D18	J Kohli
975 h$^+$	wild-type	P Thuriaux
972 h$^-$	wild-type	P Thuriaux
WT3 h$^+$	*ade 6-704*	P Thuriaux
122 h$^-$	*leu 1 his 2*	B Hall
131 h$^+$	*lys 1-131*	P Thuriaux
131 h$^-$	*lys 1-131*	P Thuriaux
WT4 h^{90}	wild-type	Iodine, this study
WT5 h^{90}	*ura 4*	Iodine, this study
WT6	mig$^-$ spo$^-$	UV from WT4
WT7	mig$^-$ *ura 4* spo$^-$	UV from WT5
WT8	mig$^-$ *ura 4* spo$^-$	UV from WT5
WT9	*ade* spo$^+$	UV from WT4
WT10	*ade* spo$^+$	UV from WT4
Schizosaccharomyces pombe/malidevorans **Hybrids**		
XL1 a	*ade 6-704* mig$^-$ spo$^-$	M Lubbers
XL1 b	*ade 6-704* mig$^-$ spo$^-$	M Lubbers
XN1 1	*ura 4* mig$^-$ spo$^-$	N Hansen
XW1 1	mig$^-$ spo$^-$	Mating, this study
XW1 2	wild-type spo$^+$	"
XW1 3	*his 2* spo$^+$	"
XW1 4	*leu 1* spo$^+$	"
XW1 5	mig$^-$ spo$^-$	"
XW1 6	*ade 6-704* spo$^+$	"
XW1 7	*ade 6-704* spo$^-$	"
XW1 8	*leu 1 his 2* spo$^-$	"
XW1 9	mig$^-$ *leu 1* spo$^-$	"
XW1 10	*ade 6-704 leu 1* spo$^-$	"
XW1 11	*ade 6-704 leu 1* spo$^-$	"
XW1 12	wild-type spo$^-$	"
XW1 13	*ade 6-704 leu 1 his 2* spo$^-$	"
XW1 14	mig$^-$ *leu 1 his 2* spo$^-$	"
XW1 15	mig$^-$ *ade 6-704 leu 1* spo$^-$	"
XW1 16	mig$^-$ *ade 6-704* spo$^-$	"
XW1 17	mig$^-$ spo$^-$	"
XW1 18	mig$^-$ spo$^-$	"
XW1 19	mig$^-$ *ade 6-704* spo$^-$	"
XW1 20	mig$^-$ *leu 1* spo$^-$	"
XW1 21	mig$^-$ *leu 1* spo$^-$	"
XW1 22	mig$^-$ *ade 6-704* spo$^-$	"
XW1 23	mig$^-$ *ade 6-704 leu 1 his 2* spo$^-$	"
XW1 24	mig$^-$ *ade 6-704* spo$^-$	"
Saccharomyces cerevisiae MD26	homothallic, diploid	R Thornton

10 ml per 200 ml medium of filter sterilized stock solutions of amino acids or bases (Table 3).

2.3. Indicator plate used for screening malate dependent mutants of *Schizosaccharomyces*

The indicator plate used to screen and check the stability of malate dependent mutants of *S. malidevorans* contained 6.7 g/l yeast nitrogen base without amino acids, 150 g/l glucose, 10 g/l malic acid, 2.2 ml/l of 10 g/l aqueous bromocresol green solution (Difco Laboratories), and 20 g/l agar. The medium was adjusted to pH 4.5 with 3 M KOH prior to the addition of agar (Ind M). Media supplemented with amino acids were prepared by the addition of stock solutions of amino acids, as for yeast minimal medium (Materials & Methods 2.2).

The indicator plate used to screen and check the stability of malate dependent mutants of *S. pombe* was identical to the indicator plate already described above except that it contained 2.5 g/l malic acid (Ind P).

2.4. Luria broth (LB)

Luria broth contained 10 g/l tryptone, 4 g/l yeast extract, and 10 g/l sodium chloride. The pH was adjusted to 7.5 with 5 M NaOH before autoclaving. Plates supplemented with (1) ampicillin were prepared by the addition of filter-sterilised ampicillin to a final concentration of 50 μg/ml; (2) tetracycline were prepared by the addition of filter sterilised tetracycline to a final concentration of 15 μg/ml.

Table 2: ***Escherichia coli*** **strains.**

STRAIN	GENOTYPE	SOURCE
JA 221	*rec* A1 *leu* B6$^-$*trp* AE5 *hsd* R$^-$M$^+$*lac* Y	M.Yamamoto
HB 101	*supE44 supF58 hsdS3*($r_B^-m_B^-$)*recA13 ara-14 proA2 lacY1 galK2 rspL20 xyl-5 mtl-1*	Massey
DH 5	*supE44 hsdR17 recA1 endA1 gyrA96 thi-1 recA1*	Massey

2.5. Bacterial minimal medium

Bacterial minimal medium contained 7 g/l dipotassium hydrogen phosphate; 2 g/l potassium dihydrogen phosphate; 1-1.5 g/l ammonium sulphate; 0.5 g/l sodium citrate; 0.1 g/l magnesium sulphate, and 2 g/l glucose. Medium supplemented with amino acids were prepared by the addition of stock solutions of amino acids as for yeast minimal medium (Materials & Methods 2.2).

2.6. Maltose assimilation medium

Maltose assimilation was tested by the method of Bridge & May (1984). The medium was yeast minimal malate (MMM) agar, with the glucose substituted with maltose. The filter sterilised maltose was added after autoclaving and cooling the meduim. For strains with auxotrophic markers amino acid stock solutions were added as for yeast minimal media (Materials & Methods 2.2). Growth or no growth was scored after the cultures had been incubated for 14 days at $30^{O}C$.

2.7. Yeast extract agar (YEA, YEAM)

This medium supports vegetative growth but inhibits conjugation and sporulation. YEA contained 5 g/l yeast extract, 30 g/l glucose, and 30 g/l agar. For crosses involving malate dependent strains, 3 g/l malic acid was added to the medium prior to the addition of agar, and the pH was adjusted to 4.8 with 3 M KOH (YEAM).

2.8. Magdala red (Phloxin-B) agar

This medium was used to differentiate haploid and diploid colonies. The media was identical to YEAM agar with the addition of filter

Table 3: Amino acids and bases for supplementing yeast and bacterial minimal media.

Amino acid or base	Concentration g/300ml stock	Final Concentration in medium (µg/ml)
L-tryptophan*	0.12	20
L-leucine	0.18	30
L-histidine*	0.12	20
L-lysine	0.18	30
Adenine	0.12	20
Uracil	0.12	20

* filter sterilised rather than autoclaved

sterilised magdala red (Difco Laboratories) at a concentration of 10 μg/ml after autoclaving.

2.9. Malt extract agar (MEA, MEAM)

This medium promotes yeast conjugation and sporulation. MEA contained 30 g/l malt extract (Difco Laboratories), and 20 g/l agar. For crosses involving malate dependent strains, 3 g/l malic acid was added prior to the addition of agar, and the pH was adjusted to 4.8 with 3 M KOH (MEAM).

2.10. Sporulation agar (SPA, SPAM)

This medium was used to promote sporulation in yeast strains. It does not contain a nitrogen source and thus does not support vegetative growth. This medium contained 7 g/l yeast nitrogen base without amino acids, 10 g/l glucose, and 30 g/l agar. For crosses involving malate dependent strains, 3 g/l malic acid was added prior to addition of agar, and the pH of the medium was adjusted to 4.8 with 3 M KOH.

2.11. Minimal malate glucose (MMG)

MMG was used to test utilisation of malate and glucose by yeast strains (Rodriguez & Thornton 1989). A modification of the medium was the addition of 0.02% asparagine, prior to autoclaving, instead of a mixture of amino acids, in order to prevent lysis in late exponential phase of *S. malidevorans* 442.

3. Mating procedure

Strains were checked for stability of auxotrophic markers on selective media and stability of mating-type by macroscopic examination of three-day old cultures on YMM plates exposed to iodine vapours (Gutz *et al.* 1974), prior to mating. YEAM plates were then streaked for single colonies and incubated at 30^{o}C for 2 days. The cultures were mass mated by mixing a loopful of the cells with a drop of sterile deionised water, on MEAM plates, and incubating for 15 hours at 25^{o}C. The matings were checked microscopically for zygotes and plated out on yeast minimal medium when many zygotes were observed. The minimal plates were incubated at 30^{o}C for 4-6 days to allow for growth of prototrophic colonies. These colonies were then patched out on SPAM plates and incubated for 3-7 days at 25^{o}C to allow sporulation to take place. Tetrad analysis (Gutz *et al.* 1974) was done as soon as azygotic asci appeared, usually after approximately 2-3 days. Random spore analysis was done by allowing the asci to break down completely, at 25^{o}C, for at least two days before harvesting the spores. The spores were incubated for 30 minutes at 18^{o}C in 30% ethanol to kill any vegetative cells (Leupold 1970). Absence of live vegetative cells was checked for by staining cells with viable stain (aqueous solution of 0.01% methylene blue, 2% sodium citrate). The spore suspension was vortexed vigorously and checked microscopically to ensure that at least 95% of the spores were dissociated from each other. The suspension was plated out to give between 100-300 spores/plate on YMM agar. The plates were incubated for 3-5 days at 30^{o}C, or until no more colonies appeared.

These plates were replica plated onto selective media and incubated for three days at 30^{O}C.

4. Ultraviolet light (UV) mutagenesis

UV mutagenesis of *Schizosaccharomyces* and *Saccharomyces cerevisiae* for isolation of malate dependent mutants was done. The cells were grown to stationary phase, centrifuged and washed once with sterile distilled water, and resuspended in sterile distilled water to a concentration of approimately 3 x 10^5 cells/ml. 0.1ml of the cell suspension was spread onto indicator plates. The plates were then exposed to UV irradiation for approximately 90 seconds. This exposure was found to kill approximately 90% of the cells. Isolation of *S. pombe* mutants required use of indicator plates containing 0.25% malic acid (IndP). For the isolation of adenine mutants, the mutagenised cells were plated out on YM media, wrapped in tin foil and incubated for 5 days at 30^{O}C. Red colonies were picked and streaked on selective media to check for adenine requirement.

5. Fermentation trials

5.1. Culture conditions in MMG

Fermentation trials in MMG of *Schizosaccharomyces* strains were done according to the method of Rodriguez & Thornton (1989), except that 200 ml of medium was used in a 250 ml flask.

The flasks were innoculated with the cells at a concentration of 5 x 10^5 cells/ml and sealed using a fermentation trap. The flasks were then incubated at 25^{O}C, shaking at 100rpm Samples (4 ml) were taken for pH measurement, HPLC analysis and estimation of the viable cell numbers, as determined by viable stain (methylene blue) (Anon 1981) with hemocytometer.

5.2. High performance liquid chromatography (HPLC) analyses

5.2.1. Preparation of samples

Samples were prepared for HPLC analysis by filtration with 0.45 µm cellulose acetate membrane filters using Swinnex filter kits (Millipore Corporation).

5.2.2. Analyses of glucose and malic acid

Glucose and malic acid were analysed by HPLC using a Shimadzu LC-4A high performance liquid chromatograph with a Shimadzu SIL-2A autosampler, a RID-2AS refractive index detector, a SPD-2AS ultraviolet (UV) detector, and a Shimadzu C-R3A Chromatopac data processor. All samples were analysed with two columns in series; a Brownlee PPH-224 cation exchange column, and a Brownlee OSS-MP reverse phase column. Filtered and degassed sulphuric acid (0.013 N) was used as the mobile phase for the column. The oven temperature was 30^{O}C and the flow rate was 0.3 ml/minute. Glucose was detected by RI detector and malate was detected by UV detector (210 nm). Columns were protected by having a Biorad Microguard Cation H guard cartridge. A two point calibration, for the RI detector, and a point to point curve calibration for the UV detector, was accomplished by injecting both a high and low standard. The high standard contained 100 g/l glucose and 3 g/l malic acid, and the low standard was a 10-fold dilution of the high standard. An HPLC chromatograph showing the separation of malate and glucose, detected by UV detector, is shown (Figure A1).

An alternative method for detection of glucose was using Diastik (Ames Laboratory). The Diastik strip is dipped into the medium and the colour of the strip, after 30 seconds, indicates the approximate concentration of glucose.

6. Enzyme assays

6.1. Preparation of cell-free extract

Cells were grown overnight in 10 ml of YMM broth and inoculated at 5×10^5 cells/ml into YMM broth. Cultures were incubated, shaking at 100 rpm, until early stationary phase at 30^{o}C, when the cells were harvested by centrifugation at 5,000 g for five minutes at 4^{o}C. Cells were washed twice with 100 ml cold deionised water and resuspended in either, 10 ml 0.05 M Tris-HCl, pH 7.5, or 0.05M phosphate buffer, pH 7.5, containing 2mM EDTA and 2mM mercaptoethanol. Following three passages through the French Press at 82.8 MPa, cellular debris was removed by centrifugation at 17,500 x *g* for 20 minutes at 4^{o}C. The supernatant was used as a cell-free extract and was stored on ice until required. Enzyme and protein assays were done on the same day as the extraction.

6.2. Hexokinase-ATP-kinase

The D-glucose-phosphorylating activity of cell-free extract was assayed by following the absorbance of NADP at 340 nm with a Gilford 260 recording spectrophotometer, temperature controlled at

$30^{O}C$, according to the method of Joshi & Jagannathan (1966). The reaction mixture consisted of 15mM D-glucose, 10mM ATP, 0.13mM NADP, 0.6 units of glucose-6-phosphate dehydrogenase, 20mM $MgCl_2$ in 20mM Tris-HCl buffer, pH 7.5. The activities of controls which lacked ATP were also measured and subtracted from the activity obtained for the enzyme. The result for this, and all other enzyme assays, was the average activity obtained from assaying at least two different cell-free extracts.

6.3. Malate dehydrogenase (MDH)

Malate dehydrogenase activity was assayed by the method of Ohshima and Sakuraba (1986) and measuring the change in absorbance of NAD at 340 nm at $25^{O}C$. The following conditions were used: O.1 M glycine-KOH buffer, 6.7 mM malic acid, 0.67 mM NAD and enzyme to make up 3.0 ml final volume of reaction mixture. Controls without NAD or without enzyme were assayed and the activities were subtracted from the activity obtained using the complete reaction mixture.

6.4. Malic enzyme

Malic enzyme activity was assayed by measuring the absorbance of NAD at 340 nm at $25^{O}C$, under the following conditions: 0.1M phosphate buffer, pH 7.5; 6.7 mM malic acid; 0.67 mM NAD; 0.17 mM $MgCl_2$, and enzyme at an appropriate dilution, to make up 3 ml final volume of reaction mixture. The temperature at which the assays were performed was $25^{O}C$. Controls were performed without NAD, without enzyme or without $MgCl_2$, as a control for MDH activity, and the activities were subtracted from the activity obtained using the complete reaction mixture.

6.5. Determination of Km values of malic enzyme

The conditions for determination of Km values for malic acid and NAD of malic enzyme were identical to those used to measure enzyme activity. A range of concentrations of malic acid (0.17 mM-20.0 mM) was used to estimate the Km value for malic acid. A range of concentrations of NAD (0.05 mM-1.67 mM)was used to estimate the Km value for NAD. The same controls as were performed for malic enzyme activity were performed for each assay in determining Km values. The reciprocal value of each initial activity (1/Vo) was plotted against the reciprocal value of the substrate concentration (1/[S]) (Lineweaver-Burke plot). The best fit line for each plot was calculated by applying the best fit line using Cricket Graph (Macintosh software). The X- intercept was the -1/Km value.

6.6. Heat inactivation of malic enzyme

Five ml of cell-free extract, diluted to a protein concentration of 1 mg/ml in 0.1 M Tris-HCl pH 7.5, was aliquoted into a universal bottle and placed in a waterbath preheated to $40^{o}C$. At appropriate time intervals, 200 μl samples were taken and placed on ice until assayed for malic enzyme activity. The decay of malic enzyme was expressed as a percentage of total activity.

6.7. Determination of protein concentration

Protein concentration of cell-free extracts was estimated using the method of Ehresmann *et al.* (1973). A standard curve was prepared by measuring the absorbances of known concentrations of bovine serum albumin (BSA). The values were plotted versus protein concentration. The values calculated were linear up to a protein concentration of at least 125 μg/ml (Figure A2).

7. Gene library construction and screening

All plasmids and gene libraries used in this study are listed in Table 4.

7.1. Preparation of large amounts of plasmid DNA

One litre of LB containing 40 μg/ml ampicillin was used to grow *E.coli* HB101 containing pFL20. Bacterial minimal medium supplemented with tryptophan was used to grow *E.coli* JA221 containing pDB262. Both cultures were grown to saturation overnight, at 37^{O}C. The cells were harvested by centrifugation at 4,000 x *g* for 10 minutes at 4^{O}C. The cells were washed twice in ice-cold STE (0.1 M NaCl, 10 mM Tris-HCl (pH 7.8), and 1 mM EDTA), and then the plasmid was extracted by the method described by Maniatis *et al.* p 89 (1982). The plasmid was further purified by cesium chloride-ethidium bromide density gradient centrifugation (Maniatis *et al.* p 93.1982) Ethidium bromide was removed by extraction with isoamyl alcohol, and the cesium chloride was removed by dialysis (Maniatis *et al.* p 94. 1982).

7.2. Preparation of *S. malidevorans* DNA

Isolation and purification of yeast DNA was done by a modification of the method used by Beach *et al* . (1982a). Prior to the cesium chloride-ethidium bromide density gradient centrifugation, 10 mg of Proteinase K (Sigma) was added to DNA in TE (10 mM Tris HCL pH 7.6, 1 mM EDTA) and incubated for 2 hours at 65^{O}C. This step

Table 4: Plasmids and genes libraries

Plasmid or Library	Selectable Marker	Source
pFL20	URA 3 *(Sacch. cereviseae)*	F. Lacroute
pDB262	LEU 2 *(Sacch. cereviseae)*	P. Nurse
pDB262 *S. pombe* library		P. Nurse
pDB262 *S. malidevorans* library		This study
pFL20 *S.malidevorans* library		This study

aided the complete resuspension of the DNA pellet. A further phenol-chloroform step was added followed by isopropanol precipitation and resuspension in 6 ml of TE. After the DNA band was removed from the ultracentrifuge tube by syringe, the ethidium bromide and cesium chloride were removed by the same methods outlined for preparation of vector DNA (Materials & Methods 7.1). Finally, high molecular weight polysaccharide was removed from the DNA by centrifugation for 1 hour at 13,000 x *g* (Raeder & Broda 1985).

7.3. Digestion of DNA by restriction endonucleases

All DNA was cleaved by restriction endonucleases and purified after cleavage as described by Maniatis *et al.* (1982) pp 104-106. In general 3-4 U of enzyme was used to digest 1μg DNA, at 37^{o}C, for 1-2 hours. The buffers used were usually the reaction buffers supplied with the enzyme.

7.4. Sodium chloride gradient centifugation

Yeast DNA was size fractionated by sodium chloride gradient centrifugation (DiLella and Woo 1987). A sodium chloride gradient was prepared by mixing 5M NaCl and 1.25M NaCl in a gradient maker and carefully allowing it to fill a SW45 ultracentrifuge tube (approximately 12ml). The DNA sample was loaded on top of the gradient, and then centrifuged for 3 hours at 36,000rpm. 1ml aliquots were collected and a sample of each run on an agarose gel, along side a sample of a BRL, 1kb ladder, to determine the size range of each sample (Plate 8). The DNA samples of the desired size range were dialysed against two changes of TE buffer (10mM Tris-HCl, pH 7.5; 1mM EDTA).

7.5. Dephosphorylation of DNA

Vector DNA was treated with calf intestinal alkaline phosphatase (CAP) according to the method outlined by Maniatis *et al.* (1982) pp133-134. Approximately 1ul of 1/10 dilutions of CAP (Boehringer Mannheim) (1.9 U) was added to 1 μg DNA. The DNA was incubated at 37^{O}C for 1 hour.

7.6. Ligation of DNA

Ligation of DNA ends was done by the method described by Schleif & Wensink (1981). 1-2μl of T4 DNA ligase (Boehringer Mannheim) was added to a total volume of 20μl ligation mixture, and this was incubated at 14^{O}C, for 18 hours.

7.7. Transformation of *E.coli*

Transformation of *E.coli* was done according to the method described by Maniatis *et al.* (1982) pp 250-251. Typically no more than 1μg of DNA was used in a transformation.

7.8. Agarose gel electrophoresis

Agarose gel electrophoresis, visualisation and photography of the DNA was done according to the method described by Maniatis *et al* (1982) pp150-163.

7.9. Transformation of *Schizosaccharomyces*

A modified version of the lithium method of Ito *et al.* (1983) was used to transform cells of *Schizosaccharomyces*. Approximately 5 x 10^{8} exponential phase cells, grown in YMM were harvested and washed once in sterile distilled water. The cells were spun at 5,000 rpm for 6 minutes and resuspended in 1ml of 0.1M LiCl-TE. The cells were incubated in LiCl-TE for 3 hours at 30^{O}C. 0.1-2μg of plasmid DNA and 40μg of carrier DNA (sonicated herring sperm DNA) was added to the cells and incubated for 30 minutes at 30^{O}C. 0.7ml of Li-TE-PEG was added and the cells incubated a further 30 minutes at 3^{0}OC. The cells were then heat shocked for 5 minutes at 45^{O}C and then spun down and resuspended in 1ml of 0.9% NaCl solution rather than TE buffer. 0.1ml amounts were spread on the appropriate minimal agar plates and these were incubated at 30^{O}C for up to 7 days.

Schizosaccharomyces was also transformed using spheroplasts (Beach *et al.* 1982a).

7.10. Rapid preparation of plasmid DNA

The rapid boiling method of Holmes and Quigley (1981) was used to prepare small amounts of plasmid DNA. This DNA was suitable for restriction enzyme digestion to calculate insert sizes. The completeness of the gene libraries was calculated using the formula of Clarke and Carbon (1976).

8. <u>Transverse alternating field electrophoresis (TAFE)</u>

The preparation of DNA samples and running of the TAFE gels was done according to the method described by Murray (1989).

8.1. Preparation of the DNA samples for TAFE

The cells were grown to late stationary phase in YMM and harvested by centrifugation at 5,000 x *g*. The cells were washed twice in chilled ET buffer (50 mM EDTA, 1 mM Tris (pH 8.0)), and resuspended in 2 ml of CES solution (20mM citrate-phosphate buffer pH 5.6, 50 mM EDTA, 0.9M sorbitol) containing 12 mg/ml zymolyase 5000T and incubated for 2 hours at 37°C. The cells were checked microscopically for the formation of protoplasts. Low melting point agarose (1.2% in 20 mM citrate-phosphate buffer pH 5.6) was melted and then equilibrated to 50°C. An equal volume of agarose was added to the cell suspension, mixed thoroughly and distributed into the mold. The mold was chilled to allow the agarose plugs to set before being pushed out into 0.25 M EDTA, 1% SDS solution and incubated at 60°C for 2 hours. The plugs were then transferred to 10 ml of NES solution (0.5 M EDTA pH 9.5, 1% SDS, 1 mg/ml proteinase K (Sigma)) and incubated overnight at 50°C. The plugs were transferred to fresh NES buffer and incubated for a further 18 hours before being transferred to ET buffer and storage at 4 °C. Before loading the plugs into the TAFE gel they were equilibrated for at least 1 hour in TAFE buffer (10 mM Tris, 0.5 mM EDTA, 4 mM acetic acid; pH 8.0).

8.2. Electrophoresis conditions

An agarose gel (0.7% in 1 x TAFE buffer) was made in the mold provided (BRL) and allowed to set at 4°C. The equilibrated plugs were then loaded and sealed into the wells with 0.7% molten agarose and the gel was again allowed to set at 4°C. The gel was then loaded into the tank in which 1 x TAFE buffer had been cooled to 14°C. The gel was electrophoresed for 120 hours using a switching interval of 60 minutes and a field strength of 2 V/cm. Staining and photography of the gels was done as described above (Materials & Methods 7.8). All the Plates of karyotypes, shown in the Results section, were either run on the same gel (no gap between lanes), or are shown along side a control (mutant 11 or *S. malidevorans* 442), that was run on the same gel.

9. Transport of radioactive substrates by *Schizosaccharomyces*

9.1. Growth and preparation of cells

Cells were grown and prepared for transport experiments as described by Osothsilp & Subden (1986a). The cells were resuspended in 0.1 M KCl pH 3.5 to 0.05 mg/ml wet weight. A sample of the resuspended cells were filtered (0.45 µ, 25mm cellulose acetate filter (Sartorius)), dried and weighed to estimate the dry weight. A ratio of 1 mg dry weight to 5 mg wet weight was consistently obtained. Cells were kept on ice until required and all experiments were completed within four hours. All uptake experiments were carried out at least twice.

9.2. Transport of ^{14}C malate

Conditions for the transport of malate were as described by Osothsilp and Subden (1986a) with modifications: 0.4ml of the cell suspension was mixed with 100µl of 0.1M KCl pH 3.5 and placed at $30^{O}C$ for 5mins.^{14}C malate at 89.5 µmol/ml, with a specific activity of 22.4 µCi/µmol, was added to the reaction mix and incubated for 15 seconds at $30^{O}C$. 0.5ml of this suspension was mixed with 5ml of cold distilled water and rapidly filtered through a cellulose acetate menbrane (Sartorius). The filter was washed twice with cold 0.1M KCl, pH 3.5 before being placed in scintillation vials containing 5 ml of cocktail (666ml toluene, 333 ml Triton-X-100, 4 gm PPO, 0.4 gm

POPOP. Nonspecific binding was controlled by boiling a portion of the cell suspension for five minutes before the addition of malate. For inhibition of malate uptake, final concentrations of 8% glucose and 1% succinate, in KCl, were used. Inhibitors were added at the same time as the radioactive substrate.

9.3. Kinetics of malate uptake

The kinetics of malate transport was done by incubating the cell suspension for 30 seconds with a range of ^{14}C malate concentrations, (1 -50 mM malate; with a specific activity of 53 μCi/μmol). Nonspecific binding was done by treating a portion of the cell suspension with 1% sodium azide for ten minutes before the addition of malate. The reciprocal value of each initial activity (1/Vo) was plotted against the reciprocal value of the substrate concentration (1/[S]) (Lineweaver-Burke plot). The best fit line for each plot was calculated by applying the best fit line using Cricket Graph (Macintosh software). The X- intercept was the -1/Km value, and the Y- intercept was the $-1/V_{max}$ value. Hill plots were also made. The Y- axis was log $(v/V_{max} - v)$, and the X- axis was log [S]. A slope greater than one suggests positive cooperativity and less than one, negative cooperativity.

9.4. Transport of ^{14}C glucose

The assay for ^{14}C glucose uptake was identical to the malate uptake assay described above. The glucose concentration added to the cell suspension was 333 μmol/ml (final concentration, 1.0%) with a specific activity of 6 μCi/μmol. Inhibition of glucose uptake was done using a final malate concentration, in KCl, of 1%. Malate was added at the same time as the radioactive substrate.

9.5. Kinetics of glucose uptake

The kinetic of glucose uptake was done as described for kinetics of malate uptake. The range of concentrations of glucose was 1 -120 mM and the specific activity was 3μCi/μmol. Eadie-Hofstee plots were made. Rate divided by substrate concentration (v/[S]), on the X- axis, was plotted against rate (v) on the Y- axis. The Y- intercept was the V_{max}, and the X- intercept was the V_{max}/Km.

Results

1. Characteristics of *S. malidevorans* 442, mutant 11 and derivatives

A mutant with a novel phenotype (mutant 11) was isolated by UV mutagenesis of *S. malidevorans* 442 by Rodriguez and Thornton (1989). The mutant was distinguished by its blue colour on indicator plate (Ind M) compared to *S. malidevorans* 442, which is green (Plate 1a, 1b and 2a, 2b). Rodriguez and Thornton (1989) found the mutant to have a pleiotropic phenotype, including strict "malate dependence", (a dependence on malic acid as well as glucose for growth), an inability to sporulate, a lowered ability to utilise glucose, and a lower maximum cell number than the wild-type when grown in liquid medium. The mutation is designated as mig$^-$ (loss of malate independent growth).

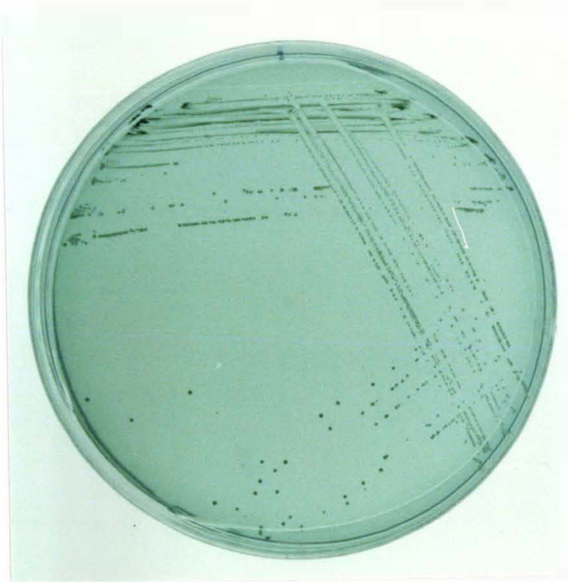
In this study further characterisation of the wild-type strain, *S. malidevorans* 442 and mutant 11 was undertaken. HPLC analysis of fermentation trials in MMG confirmed a previous study (Rodriguez and Thornton 1989) showing that malate was co-utilised with glucose in both the wild-type and mutant 11 (Figures 1 and 2). The pH was recorded during fermentations by *S. malidevorans* 442 and mutant 11 and were found to differ significantly. The medium during the growth of *S. malidevorans* 442 became more acid whereas the medium during the growth of mutant 11 became more alkaline (Figures 1 and 2). Mutant 11 was found to be sensitive to medium with a pH greater than 5.2 (Rodriguez and Thornton 1989),

Plate 1a: *S. malidevorans* 442 grown on indicator plate (Ind M).

Plate 1b: Single colonies of *S. malidevorans* 442 on Ind M.

Plate 2a: *S. malidevorans* mutant 11 grown on indicator plate (Ind M).

Plate 2b: Single colonies of *S. malidevorans* mutant 11 on Ind M.



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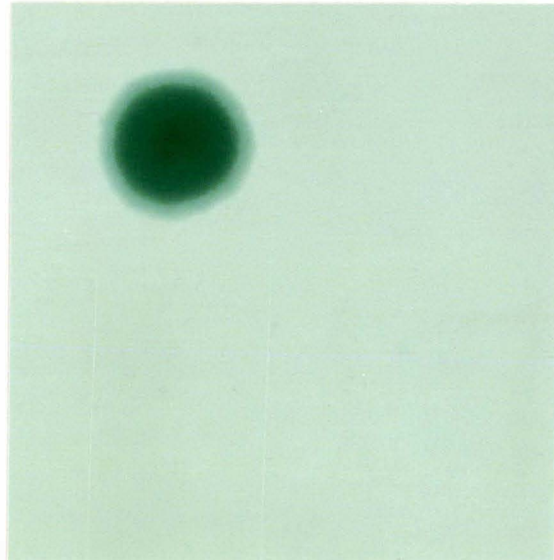

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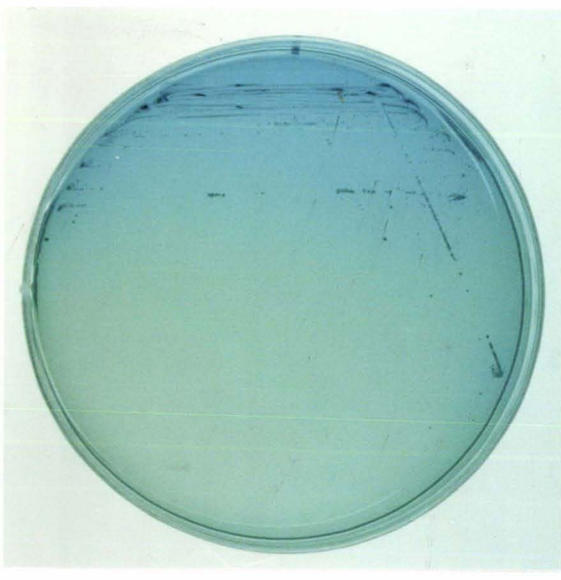


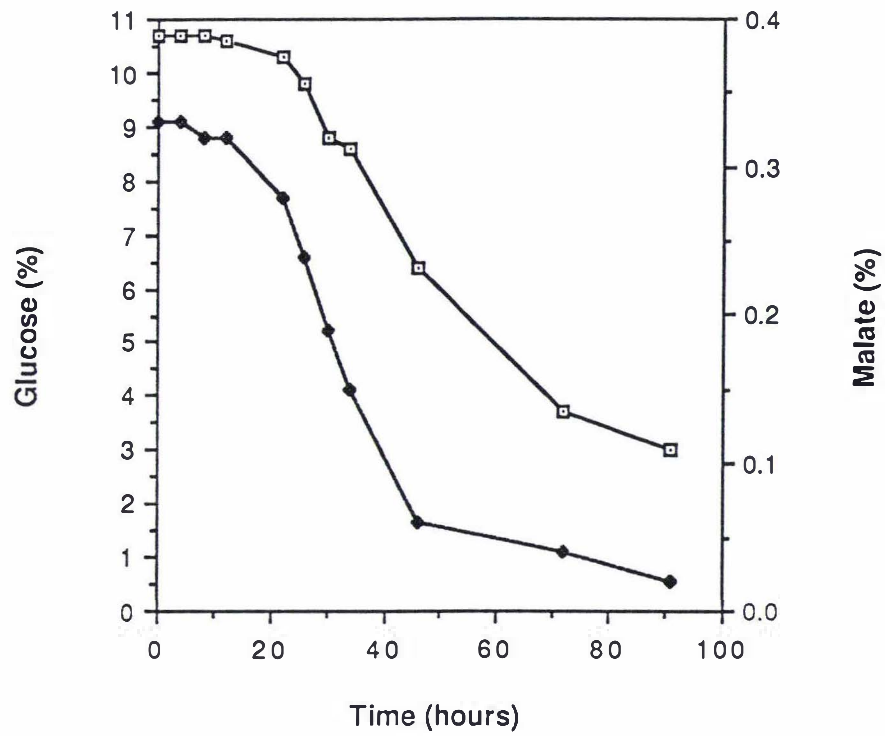
a

b

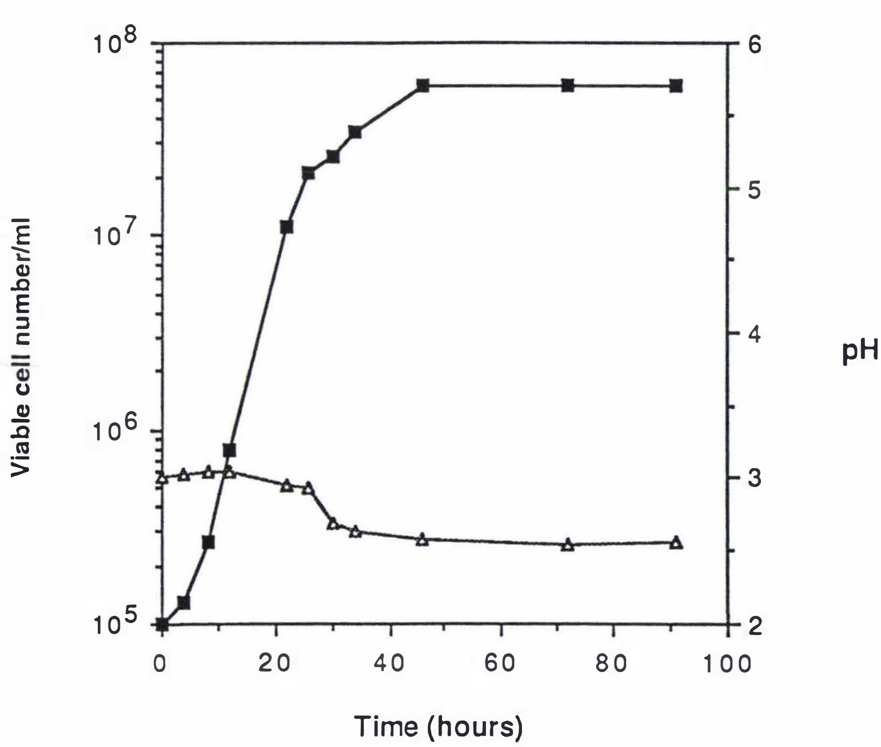
Figure 1: Fermentation trial of *S. malidevorans* 442 in MMG.

A Malate, ◆, and Glucose, □.

B Cell number, ■, and pH, Δ.



11
10
9
8
7
6
5
4
3
2
1
0
Glucose (%)
0.4
0.3
0.2
0.1
0.0
Malate (%)
0
20
40
60
80
100
Time (hours)

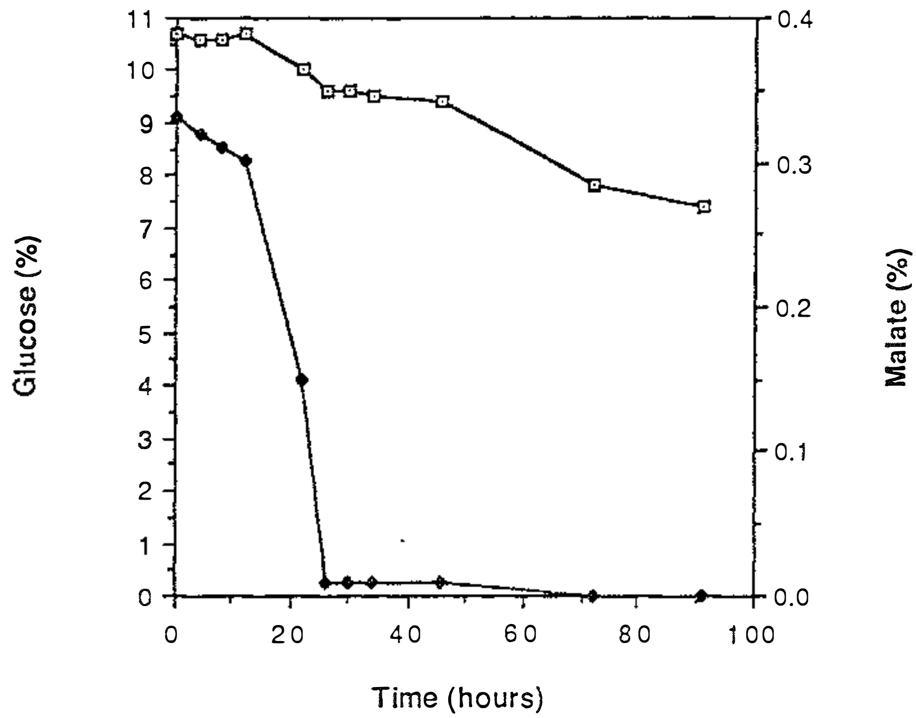


10^8
10^7
10^6
10^5
Viable cell number/ml
6
5
4
3
2
pH
0
20
40
60
80
100
Time (hours)

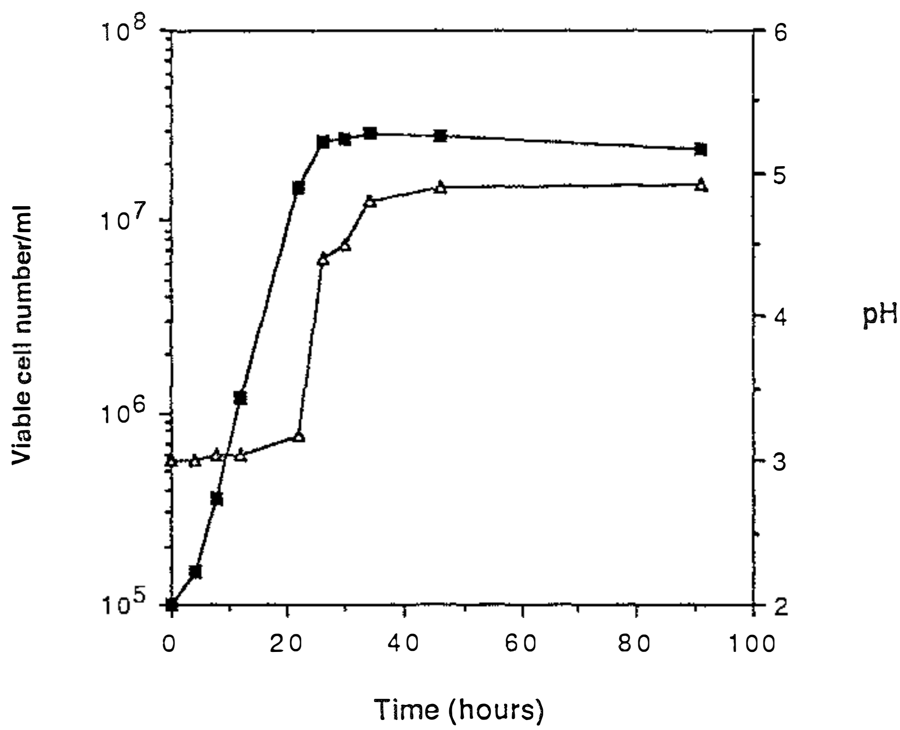
Figure 2: Fermentation trial of *S. malidevorans* mutant 11 in MMG.

A Malate, ◆, and Glucose, □.

B Cell number, ■ , and pH, Δ.



Glucose (%)
Malate (%)
Time (hours)



Viable cell number/ml
pH
Time (hours)

whereas the wild-type was able to grow up to at least pH 8.0. All these criteria were used to compare *S. malidevorans* 442 and mutant 11 with other strains of interest (Tables 5, 6 and 7).

Spontaneous reversion of malate dependence in mutant 11, i.e. the ability to grow on glucose as sole carbon source, was approximately 1 in 10^7 cells screened, which was lower than the previously reported reversion rate (Rodrigeuz and Thornton 1989). Two types of revertant colonies were observed at approximately the same frequency. Revertants of the first type, class1, were malate independent, had regained the ability to sporulate, and were green on indicator plate containing 1% malate, like *S. malidevorans* 442 (Table 5). The colonies, which appeared on YM plates after 2-3 days, were the same size as *S. malidevorans* 442 colonies and the cells were indistinguishable from *S. malidevorans* 442 cells by microscopic examination. The second type of revertants, class 2, were also malate independent but were unable to sporulate and were blue on indicator plate containing 1% malate (IndM). The colonies appeared after 5-7 days on YM plates, were very small and slow growing. Cells from these colonies were small and slightly rounded, very like cells of mutant 11. Faster growing, blue colonies appeared on IndM plates streaked with class 2 revertants. These cells were malate dependent and nonsporulating. The two revertant types were further characterised by comparing the behaviour in fermentation trials in MMG (Table 5).

S. pombe/S. malidevorans hybrids with the mutation(s) were identified by whether or not they required malate for growth,

Table 5: Phenotypic characteristics of *S. malidevorans* 442, mutant 11, and two classes of revertants of mutant 11

Characteristic	Strains			
	442	11	Revertant class 1	Revertant class 2
Malate dependence	Independent	Dependent	Independent	Independent
Colour [1]	Green	Blue	Green	Blue
Sporulation	Positive	Negative	Positive	Negative
Cell number *	6×10^7	3×10^7	6×10^7	2×10^7
pH *	2.6	4.6	2.6	2.6
Glucose utilisation *	70-80%	10-20%	70-80%	70-80%
Malate utilisation *	90%	99%	90%	90%
Sensitivity to pH above 5.2 [2]	No	Yes	No	Not Done

[1] Colour on indicator plate containing 1% malate

* After fermentation in MMG

[2] Determined by failure to grow on plates above pH 5.2

Table 6: Phenotypic characteristics of two *S. pombe* strains and two malate dependent *S. pombe/S. malidevorans* hybrids

Characteristic	Strains			
	S. pombe WT 3	XL1 b	*S. pombe* 122	XW1 15
Malate dependence	Independent	Dependent	Independent	Dependent
Colour [1]	Green	Blue	Green	Blue
Sporulation	Negative	Negative	Negative	Negative
Cell number *	7×10^7	2×10^7	5×10^7	2×10^7
pH *	2.6	4.6	2.6	4.6
Glucose utilisation *	70-80%	20%	70-80%	20%
Malate utilisation *	90%	90%	90%	90%
Sensitivity to pH above 5.2 [2]	No	Yes	No	Yes

[1] Colour on indicator plate containing 0.25% malic acid

* After fermentation in MMG

[2] Determined by failure to grow on plates above pH 5.2

colour on indicator plate (IndP) and whether or not they could sporulate. In order to investigate whether these characteristics indicated the presence of the entire mutant 11 phenotype in malate dependent *S. pombe*/*S. malidevorans* hybrids two strains were investigated further. A malate dependent hybrid, XL1 b (*ade 6-704* mig$^-$), from a cross between mutant 11 and *S. pombe* WT 3 (*ade 6-704*), and a malate dependent hybrid, XW1 15 (*ade 6-704, leu 1*, mig$^-$), from a cross between *S. pombe* 122 (*his 2, leu 1*), and XL1 b (*ade 6-704*, mig$^-$), (described in section 5.1.), were characterised further, along with the *S. pombe* parents. (Table 6). While the malate dependent hybrids do not appear very similar to the *S. pombe* parents they are very similar to mutant 11 (Table 5). These data indicate that the pleiotropic mutant phenotypes have co-segregated through two successive crosses with *S. pombe* strains.

2. Isolation of *S. pombe* malate dependent mutants

The Ind M medium does not differentiate mutant 11 and *S. pombe* strains clearly, so a modified plate (Ind P) (Lubbers 1987) was used to screen for malate dependent mutants of *S. pombe*. Lubbers (1987) failed to isolate malate dependent mutants from a heterothallic *S. pombe* strain. An attempt to isolate *S.pombe* malate dependent mutants from homothallic strains was made because it appeared from the genetic analysis that these mutants showed an association between homothallism and malate dependence. *S. pombe* WT 4 and *S. pombe* WT 5 (*ura 4*) cells were mutagenised by exposure to UV. The time of exposure allowed for

90% kill. Approximately 4,500 surviving *S. pombe* WT 4 cells were screened, and one malate dependent mutant WT 6 was isolated. Approximately 7,500 surviving *S. pombe* WT 5 cells were screened, yielding two malate dependent mutants WT 7 and WT 8. Thus malate dependent mutants can be isolated from more than one *S. pombe* strain provided that it is homothallic.

The difference in colour between *S. pombe* WT 4 and mutant WT 6 on Ind P (Plate 3a, 3b and 4a, 4b) was as marked as the difference between *S. malidevorans* 442 and mutant 11 (Plate 1a, 1b and 2a, 2b).

Both WT 7 and WT 8 had reduced viability on YMM and particularly on MEA agar used in crosses. Cells from both mutants grown on these plates appeared swollen when examined microscopically. WT 6 was chosen for further study because it had no growth requirements other than malate and glucose and was, therefore, similar to mutant 11. Revertants of WT 6 appeared with a frequency of 2 per 10^7 cells screened and were similar to the class 1 revertants of mutant 11. No class 2 revertants were found.

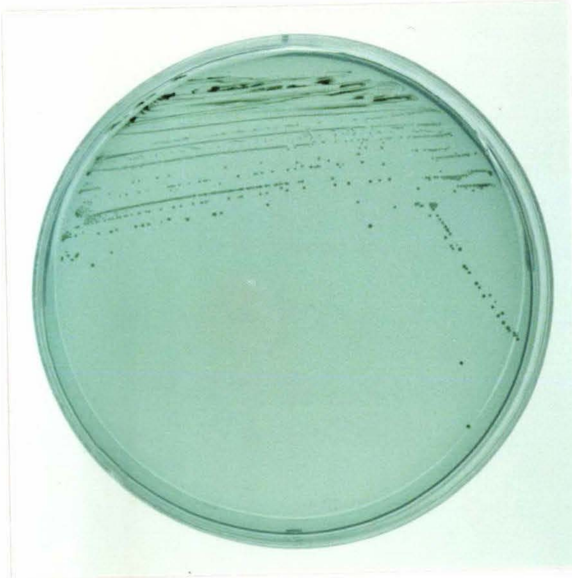
The characteristics of the malate dependent mutants of *S. pombe,* described above, suggest that these mutants are very similar to those isolated from *S. malidevorans* 442. Fermentation trials of the *S. pombe* mutant and non-mutant strains were carried out and compared to the HPLC analysis of *S. malidevorans* 442 and mutant 11.

Plate 4a: *S. pombe* mutant WT 6 grown on indicator plate (Ind P).

Plate 4b: Single colonies of *S. pombe* mutant WT 6 on indicator plate (Ind P).

Plate 3a: *S. pombe* WT 4 grown on indicator plate (Ind P).

Plate 3b: Single colonies of *S. pombe* WT 4 on indicator plate (Ind P).



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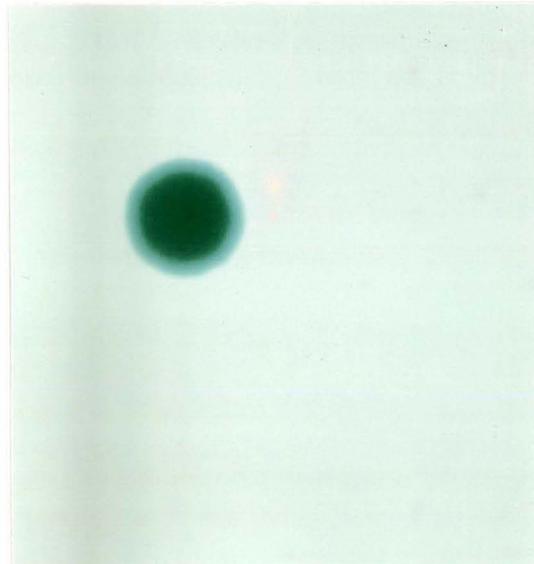

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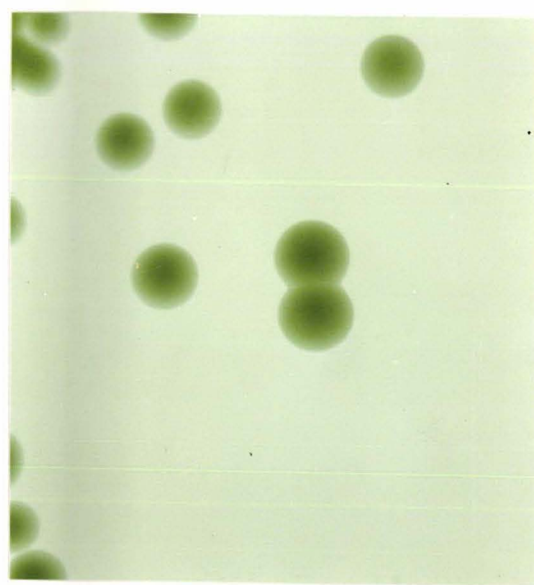
a

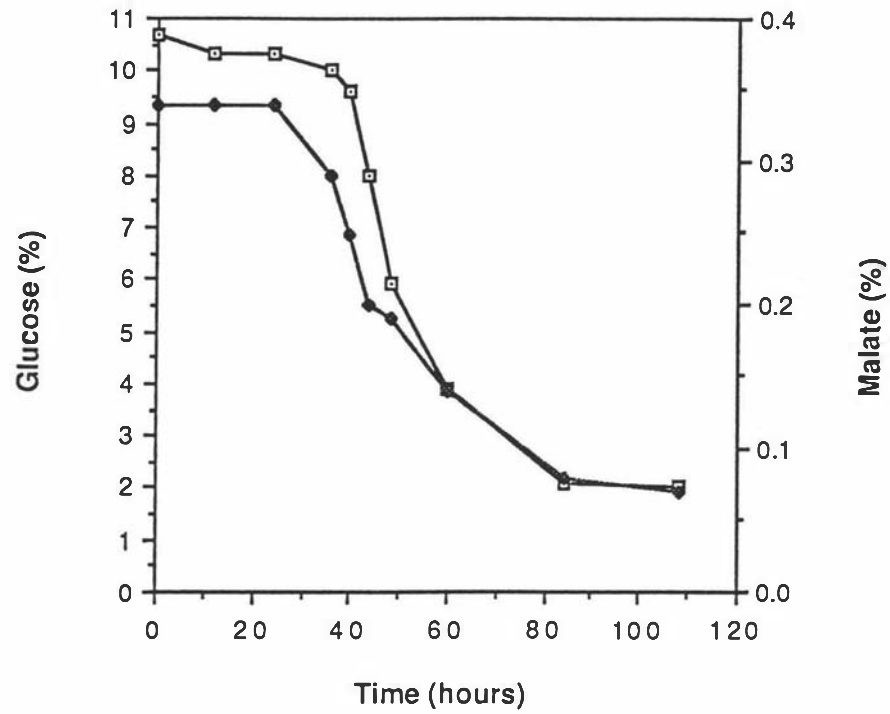


b

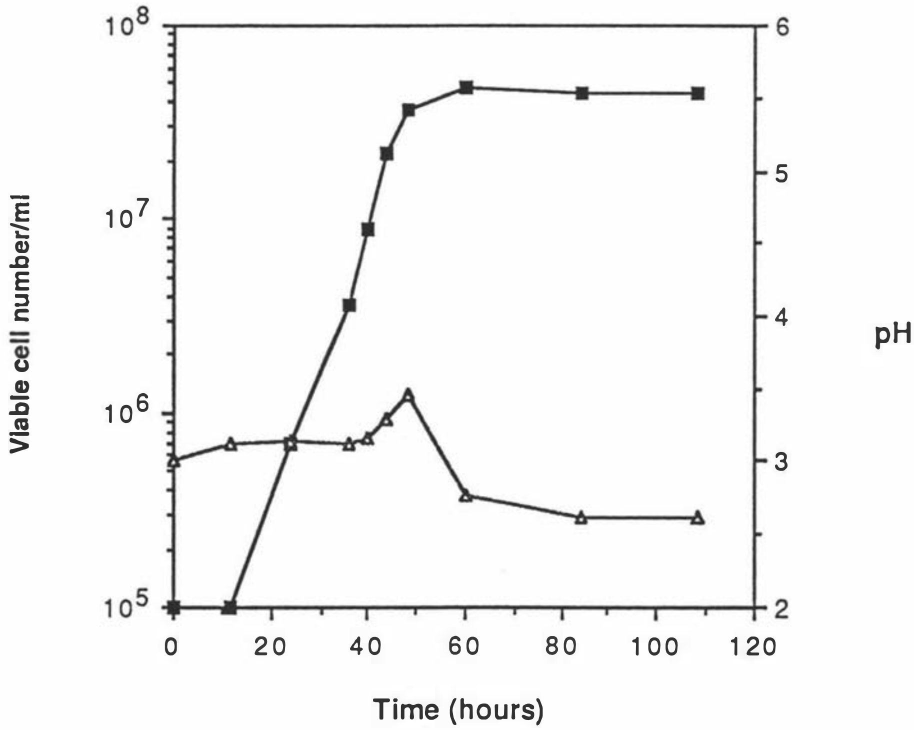
Figure 3: Fermentation trial of *S. pombe* WT 4 in MMG.

A Malate, ◆, and Glucose, □.

B Cell number, ■ , and pH, Δ.



Glucose (%)
Malate (%)
Time (hours)
0
20
40
60
80
100
120
0.0
0.1
0.2
0.3
0.4

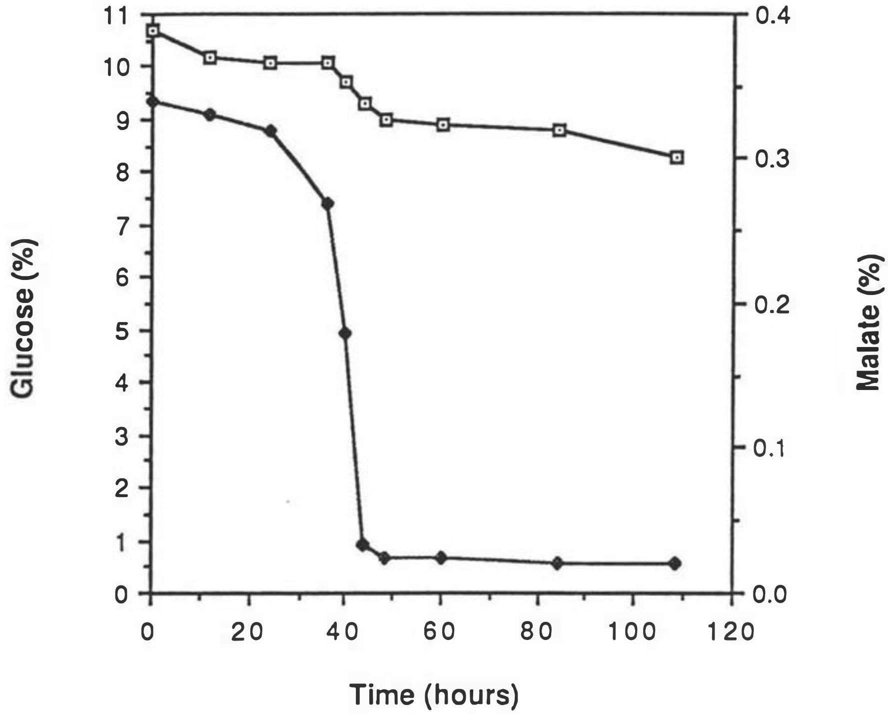


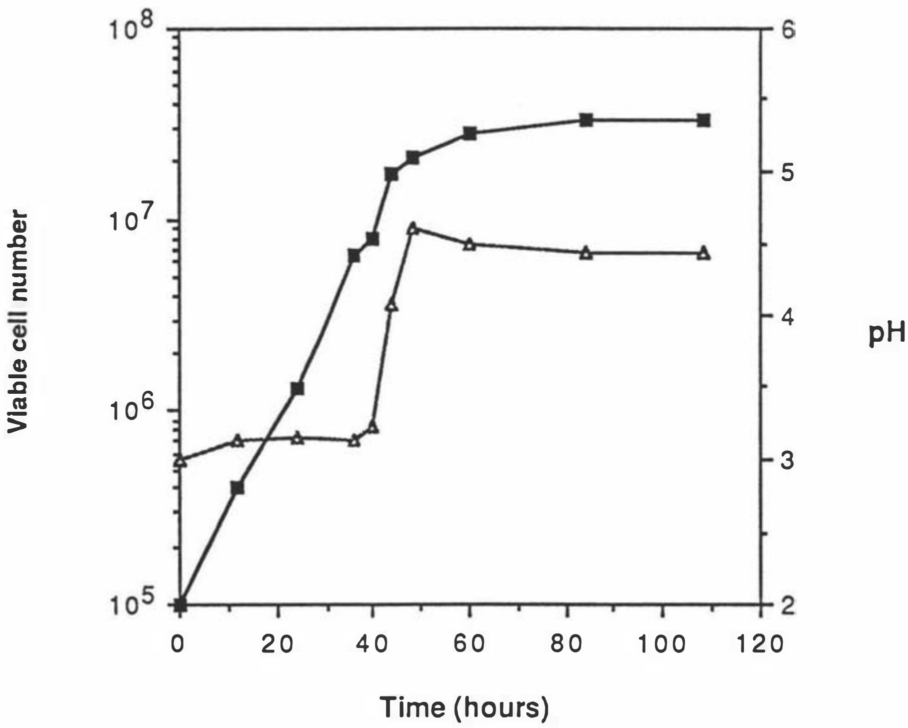
Viable cell number/ml
pH
Time (hours)
10^5
10^6
10^7
10^8

Figure 4: **Fermentation trial of *S. pombe* malate dependent mutant, WT 6, in MMG.**

A **Malate, ◆, and Glucose, □ .**

B **Cell number, ■ , and pH, Δ.**


Glucose (%)
Malate (%)
Time (hours)
0
20
40
60
80
100
120
0.0
0.1
0.2
0.3
0.4


Viable cell number
pH
Time (hours)
10^5
10^6
10^7
10^8

The fermentation patterns of *S. malidevorans* 442 and *S. pombe* WT 4 were similar in MMG, differing only in the amount of malate utilised (Figure 1, 3). *S. malidevorans* 442 utilised approximately 90% of the malate by the end of the fermentation. *S. pombe* WT 4 used approximately 73%. The characteristics of WT 6 and *S. pombe* WT 4 and a revertant of WT 6 are shown in Table 7. WT 6 was able to utilise nearly all the malate in the medium and its ability to use glucose was reduced. Comparison of utilisation of glucose and malate and pH profiles of WT 6 and mutant 11 shows the similarity of mutants from the two species (Figures 2,4). Comparison of other characteristics also demonstrated a great degree of similarity between WT 6 and mutant 11 (Table 5 and 7). One difference between the two mutants was the time taken to utilise all the malate in MMG. Mutant 11 utilised all the malate in MMG in approximately 25 hours whereas WT 6 took approximately 45 hours (Figures 2 and 4). The class 1 revertant of WT 6, like the class 1 revertant of mutant 11, appears to have regained all wild-type functions (Table 7).

3. U.V mutagenesis of *Saccharomyces cerevisiae*

Malate dependent mutants can only be isolated from homothallic strains of *S. pombe*. In order to ascertain whether a similar association between homothallism and malate dependence existed in *Sacch. cereviseae*, the homothallic strain MD26 (Thornton 1985) was mutagenised. The cells were plated on IndM medium and exposed to UV. Nonmutagenised colonies were yellow on IndM plates. The time of exposure allowed for 90% kill. Approximately

Table 7: Phenotypic characteristics of *S. pombe* WT 4, a malate dependent mutant WT 6, and a class 1 revertant of WT 6.

Characteristic	Strains		
	S. pombe WT 4	WT 6	Revertant class 1
Malate dependence	Independent	Dependent	Independent
Colour [1]	Green	Blue	Green
Sporulation	Positive	Negative	Positive
Cell number *	6×10^7	2×10^7	7×10^7
pH *	2.6	4.6	2.6
Glucose utilisation *	70-80%	20%	70-80%
Malate utilisation *	65-75%	90-95%	65-75%
Sensitivity to pH above 5.2 [2]	No	Yes	No

[1] Colour on indicator plate containing 0.25% malic acid

* After fermentation in MMG

[2] Determined by failure to grow on plates above pH 5.2

18,500 surviving *Sacch. cerevisiae* cells were screened. Three hundred and fifteen colonies were selected by their blue or green colour on IndM. None of these colonies were malate dependent. Two adenine mutants were obtained from this mutagenesis.

4. Maltose assimilation by *Schizosaccharomyces*

There are conflicting reports whether or not *S. malidevorans* is able to assimilate maltose (Rankine and Fornachon 1964, Barnett *et al.* 1983). In addition, Bridge and May (1988) reported that mating-type affected maltose assimilation in *S. malidevorans,* ie. homothallic *S. malidevorans* can assimilate maltose but heterothallic lines cannot. The ability of *S. malidevorans* to assimilate maltose was, therefore, investigated in this study.

S. malidevorans 442 is homothallic and, like *S. pombe* homothallic strains, produces zygotic asci with high frequency. Heterothallic lines were isolated from *S. malidevorans* 442 by selection for non-sporulating colonies by exposure to iodine vapours, and confirmed by crosses with mating-type strains of *S. pombe.*

S. malidevorans 442 and mutant 11 were tested and were unable to assimilate maltose. Heterothallic lines of *S. malidevorans* 442 were also unable to assimilate maltose. This contrasts with the findings of Bridge and May (1988).

Both homothallic and heterothallic *S. pombe* strains were tested, including WT 6, WT 7 and WT 8, and were all able to assimilate maltose.

5. Genetic Analysis

5.1. Crosses between mutant 11 and *S. pombe* auxotrophic strains

Genetic analysis of crosses between mutant 11 and *S. pombe* strains were hindered by low spore viability and abberrant marker segregation ratios (Rodriguez and Thornton 1989). Doubly marked strains were obtained from crossing mutant 11 and *S. pombe* h^+ *ade 6-704* (Lubbers 1987). One of these strains, XL1 b (*ade 6-704*, mig$^-$) was crossed with *S. pombe* 122 h^- (*leu 1, his 2*) in order to obtain a hybrid with increased *S. pombe* genetic background and thereby improve the chances of obtaining usable genetic data.

The complete data showing the number of representatives of the 32 possible spore classes from the cross outlined above is given in Table 8. The segregation of markers from the random spore analysis is summarised in Table 9. The expected ratios of 1:1, indicating independent assortment of genes, was not seen for any auxotrophic marker or for the ability to sporulate (spo$^+$, spo$^-$).

Tetrad dissection of fifty asci containing four spores each yielded a total of twenty six viable spores, or 13% viability. The number of viable spores per ascus was never greater than one. The

Table 8: Genotypes/phenotypes of 819 spores from the cross XL1 b (*ade 6-704* mig^-) and *S. pombe* 122 (*leu 1, his 2*).

ade^+ mig^+ leu^+ his^+ spo^+	138	ade^- mig^+ leu^+ his^+ spo^+	157
ade^+ mig^+ leu^+ his^+ spo^-	7	ade^- mig^+ leu^+ his^+ spo^-	19
ade^+ mig^+ leu^+ his^- spo^+	1	ade^- mig^+ leu^+ his^- spo^+	0
ade^+ mig^+ leu^+ his^- spo^-	10	ade^- mig^+ leu^+ his^- spo^-	24
ade^+ mig^+ leu^- his^+ spo^+	30	ade^- mig^+ leu^- his^+ spo^+	40
ade^+ mig^+ leu^- his^+ spo^-	9	ade^- mig^+ leu^- his^+ spo^-	18
ade^+ mig^+ leu^- his^- spo^+	0	ade^- mig^+ leu^- his^- spo^+	4
ade^+ mig^+ leu^- his^- spo^-	42	ade^- mig^+ leu^- his^- spo^-	121
ade^+ mig^- leu^+ his^+ spo^+	0	ade^- mig^- leu^+ his^+ spo^+	0
ade^+ mig^- leu^+ his^+ spo^-	39	ade^- mig^- leu^+ his^+ spo^-	88
ade^+ mig^- leu^+ his^- spo^+	0	ade^- mig^- leu^+ his^- spo^+	0
ade^+ mig^- leu^+ his^- spo^-	2	ade^- mig^- leu^+ his^- spo^-	10
ade^+ mig^- leu^- his^+ spo^+	0	ade^- mig^- leu^- his^+ spo^+	0
ade^+ mig^- leu^- his^+ spo^-	9	ade^- mig^- leu^- his^+ spo^-	12
ade^+ mig^- leu^- his^- spo^+	0	ade^- mig^- leu^- his^- spo^+	0
ade^+ mig^- leu^- his^- spo^-	8	ade^- mig^- leu^- his^- spo^-	31

Table 9: Segregation of markers from random spore analysis of 819 spores from the cross: XL1 b (*ade 6*, mig^{-}) and *S. pombe* 122 (*leu 1, his 2*)

Phenotype	adenine	histidine	malate	leucine	sporulation
+	295	566	620	495	370
-	524	253	199	324	449

Table 10: Linkage analysis of markers from the cross; *S. pombe/S. malidevorans* XL1 b (*ade 6-704*, mig^{-}) and *S. pombe* 122 (*leu 1, his 2*).

P his^{+} leu^{+} 448	R his^{+} mig^{+} 418	R his^{+} ade^{+} 232
R his^{+} leu^{-} 118	P his^{+} mig^{-} 148	P his^{+} ade^{-} 334
R his^{-} leu^{+} 47	P his^{-} mig^{+} 202	P his^{-} ade^{+} 63
P his^{-} leu^{-} 206	R his^{-} mig^{-} 51	R his^{-} ade^{-} 190
20.2 map units	57.3 map units	51.5 map units
Linked	Unlinked	Unlinked
R ade^{+} leu^{+} 197	P ade^{+} mig^{+} 237	R leu^{+} mig^{+} 356
P ade^{+} leu^{-} 98	R ade^{+} mig^{-} 58	P leu^{+} mig^{-} 139
P ade^{-} leu^{+} 298	R ade^{-} mig^{+} 383	P leu^{-} mig^{+} 264
R ade^{-} leu^{-} 226	P ade^{-} mig^{-} 141	R leu^{-} mig^{-} 60
51.6 map units	53.9 map units	50.8 map units
Unlinked	Unlinked	Unlinked

P = progeny of the parental type
R = progeny of the recombinant type

genotypic/phenotypic classes for these spores were in very similar proportions to those found in the random spore analysis, indicating that the random spore analysis is comparable.

Low spore viability, resulting in missing spore classes, may affect the validity of linkage analysis. The pairwise analysis of markers (Table 10) indicates linkage between *leu 1* and *his 2*, markers known to be linked in *S. pombe* on chromosome 2. The ratios between leu^+ and leu^-, and his^+ and his^- spores were 1.5:1 and 2.2:1 respectively, suggesting that significant numbers of all alleles were present. The *ade 6-704* mutation maps on chromosome 3 in *S. pombe* and is, therefore, unlinked to either *his 2* or *leu 1*. In this cross *ade 6-704* was found to be unlinked to either auxotrophic marker. The ratio between ade+ and ade- spores was 1:1.8. Linkage was not detected between malate dependence and any of the auxotrophic markers in this cross. However, the ratio between mig+ and mig- spores was 3.1:1. This ratio is greater than the ratios of the markers for which the positions are known. A large proportion of the mig- spores were apparently non-viable, which is likely to distort any genetic analysis involving this marker.

All malate dependent mutants obtained in this and other studies (Rodriguez and Thornton 1989, Lubbers 1987) were unable to sporulate. Results from this and other crosses showed that when malate dependent mutants or malate dependent *S. pombe/S. malidevorans* hybrids were crossed with heterothallic *S. pombe* strains a proportion of the progeny were homothallic, indicating that the malate dependent parents of these crosses were

Table 11: Results of testing the mating-type of progeny from crosses between *S. malidevorans/S. pombe* hybrids

Strains	Number of strains that; successfully crossed with:		Number of strains in which reversion was accompanied by the ability to sporulate
	S. pombe h^+	*S. pombe* h^-	
12 malate dependent hybrids[1]	11	12	12
5 malate independent hybrids (spo-)[2]	5	0	0

1 Unable to sporulate.

2 Heterothallic (h+, h-).

genotypically homothallic. To check the mating-type of the progeny of this cross, twelve malate dependent and five malate independent, non-sporulating progeny were crossed with heterothallic strains of *S. pombe* (975 h^{+} & 972 h^{-} ; h^{+} *lys 1* & h^{-} *lys 1*). Mating was detected by microscopic visualisation of zygotic asci on MEAM and formation of prototrophic colonies on minimal media (Table 11). Eleven of twelve malate dependent hybrids mated with both h^{+} and h^{-} strains. When malate independent revertants were isolated from them, all twelve sporulated in pure clones. The results strongly suggest that all malate dependent strains are homothallic. The five malate independent strains were heterothallic, i.e. they mated with only the h^{+} strains and did not sporulate in pure clones.

All malate independent progeny were designated as either homothallic or heterothallic depending on sporulation ability and, of the 620 malate independent spores, 370 were homothallic (Table 12). All malate dependent progeny were assumed to be homothallic. A pairwise analysis was made of each of the auxotrophic markers with the mating-type locus. The ratio of homothallic (hom) to heterothallic (het) spores was 1.2:1. These data indicate that *leu 1* and *his 2* are linked to the mating-type locus (Table 12). Pairwise analysis of malate dependence and mating-type suggests they that they are not linked (Table 12), however, due to the unequal ratio of mig^{+} to mig^{-} spores this interpretation is inconclusive.

Table 12: Linkage analysis of mating-type with markers from the cross: *S. pombe/S.malidevorans* XL1 b (*ade 6-704*, mig⁻) and *S. pombe* 122 (*leu 1, his 2*).

P	his⁺	hom	513	R	ade⁺	hom	227
R	his⁺	het	53	P	ade⁺	het	58
R	his⁻	hom	56	P	ade⁻	hom	342
P	his⁻	het	197	R	ade⁻	het	182
13.3 map units				49.9 map units			
Linked				Unlinked			
R	leu⁺	hom	435	R	mig⁺	hom	370
P	leu⁺	het	60	P	mig⁺	het	250
P	leu⁻	hom	134	P	mig⁻	hom	199
R	leu⁻	het	190	R	mig⁻	het	0
23.7 map units				45.2 map units			
Linked				Unlinked			

P = progeny of the parental type
R = progeny of the recombinant type
hom = homothallic
het = heterothallic

Table 13: Genotypes/phenotypes of 147 spores from the cross XW1 9 (*leu 1*, mig$^-$) and *S.pombe* WT 2 (*ura 4*)

mig$^+$ leu$^+$ ura$^+$ hom	42	mig$^-$ leu$^+$ ura$^+$ hom	14
mig$^+$ leu$^+$ ura$^+$ het	19	mig$^-$ leu$^+$ ura$^+$ het	0
mig$^+$ leu$^+$ ura$^-$ hom	11	mig$^-$ leu$^+$ ura$^-$ hom	0
mig$^+$ leu$^+$ ura$^-$ het	0	mig$^-$ leu$^+$ ura$^-$ het	0
mig$^+$ leu$^-$ ura$^+$ hom	8	mig$^-$ leu$^-$ ura$^+$ hom	44
mig$^+$ leu$^-$ ura$^+$ het	0	mig$^-$ leu$^-$ ura$^+$ het	0
mig$^+$ leu$^-$ ura$^-$ hom	1	mig$^-$ leu$^-$ ura$^-$ hom	0
mig$^+$ leu$^-$ ura$^-$ het	8	mig$^-$ leu$^-$ ura$^-$ het	0

hom = homothallic
het = heterothallic

A cross was made between a malate dependent hybrid XW1 9 (mig$^-$, *leu 1*) and *S. pombe* WT 2. It was hoped that XW1 9 would be more compatible with *S. pombe*, resulting in improved spore viability. The complete data are shown in Table 13. Tetrad dissection of 20 asci yielded only 14 viable spores, or a viability of 17.5%, no higher than the cross, XL1 b (*ade 6-704*, mig$^-$) crossed with *S. pombe* 122 *h*$^-$ (*leu 1, his 2*), described above. The segregation pattern of auxotrophic markers from the random spore analysis was not the expected 1:1 ratio for some of the markers (Table 14). However, segregation of leu$^+$ to leu$^-$ spores was 1:1 and segregation of mig$^+$ to mig$^-$ spores was 1.5:1. Pairwise analysis between *leu 1* and mig suggested that the two loci may be linked (Table 15).

Progeny with uracil auxotrophy were under-represented and progeny that required both malate and uracil for growth were not found (Table 15). The ratio of ura$^+$ to ura$^-$ spore was 6.4:1. Heterothallic spores were also under-represented, with a ratio of heterothallic to homothallic spores of 1:4.4. Linkage analysis between the under-represented markers and *leu 1* or malate dependence was done (Table 15). These data did not show linkage between the mating-type locus and *leu 1*, although they are, in fact, linked in *S. pombe*. Lack of apparent linkage is likely to be a consequence of the under-represented spore classes.

Table 14: Segregation of markers from random spore analysis of the cross: XW1 9 (*leu 1*, mig⁻) and *S. pombe* WT 2 (*ura4*)

	malate	leucine	uracil	mating-type
+	89	75	127	120
–	58	72	20	27

+ = independent, or homothallic for mating-type

– = dependent, or heterothallic for mating-type

Table 15: Pairwise analysis of progeny from the cross XW1 9 (*leu 1*, mig⁻) and *S. pombe* WT 2 (*ura 4*)

P	mig^+ hom	62
R	mig^+ het	27
R	mig^- hom	58
P	mig^- het	0
42.2 map units		
Unlinked		
R	leu^+ hom	67
P	leu^+ het	19
P	leu^- hom	53
R	leu^- het	8
51.0 map units		
Unlinked		
P	mig^+ leu^+	72
R	mig^+ leu^-	17
R	mig^- leu^+	14
P	mig^- leu^-	44
25.2 map units		
Linked		
R	mig^+ ura^+	69
P	mig^+ ura^-	20
P	mig^- ura^+	58
R	mig^- ura^-	0
46.9 map units		
Unlinked		

P = progeny of the parental type

R = progeny of the recombinant type

5.2. Crosses between *S. pombe* malate dependent mutants and *S. pombe* auxotrophic strains

Three malate dependent mutants were obtained from two different *S. pombe* strains. WT 6 was obtained from *S. pombe* WT 4, a homothallic derivative of *S. pombe* 975 h^+. WT 7 and WT 8 were obtained from *S. pombe* WT 5, a homothallic derivative of *S. pombe ura 4* h^+. There should be no incompatibility problems with crosses done between members of the same species, and it may, therefore, be possible to obtain useful genetic data from crosses with these mutants. All three malate dependent mutants were crossed with *S. pombe* WT 3 h^-*(ade 6-704)* and *S. pombe* 122 h^- *(leu 1, his 2)*. WT 6 was also crossed with *S. pombe* WT 2 h^- *(ura 4)*.

During crosses, cells were checked for zygote formation on MEA plates, but none were seen. In each cross, but particularly in the two crosses involving the mutants with uracil auxotrophy, cell death occurred on MEA plates. Similarly, no obvious zygote formation had been observed in any crosses involving malate dependent mutants from *S. malidevorans* 442 (Lubbers 1987, Hansen personal communication). In contrast, abundant zygotes were seen in a control cross between *S. pombe* h^- 972 and *S. pombe* h^+ 975. Diploid formation was tested by formation of colonies on minimal plates (Table 16). WT 7 was found to have a high reversion frequency, therefore no further crosses were done with this mutant.

Table 16: Formation of prototrophic colonies on yeast minimal media from crosses involving *S. pombe* malate dependent mutants and *S. pombe* auxotrophic strains.

Strains	WT 3	WT 2	122	Control*
WT 6	+	+	ND	-
WT 7	+	ND	+	+
WT 8	+	ND	+	-
122	+	-	-	-
WT 3	-	+	+	-
WT 2	+	-	-	-

* Control column indicates colonies formed when malate dependent strains are streaked out on yeast minimal medium without crossing.

\+ = diploids or revertants
\- = no diploids or revertants

ND: Not Done

Colonies on minimal plates from crosses involving WT 6 and WT 8 were very small and slow growing in comparison to the diploids usually seen in *S. pombe* crosses. The numbers of colonies did not differ between crosses involving malate dependent strains and those not involving these strains. Attempts to find azygotic asci from diploids streaked on SPAM for tetrad analysis failed, although abundant zygotic asci were seen, indicating that the colonies on minimal plates were probably haploid prototrophic progeny from the cross rather than diploids. All zygotic asci dissected yielded at least two prototrophic spores.

To obtain diploids before commitment to meiosis, the crosses were repeated and streaked out on minimal plates at 10, 12, 14 and 16 hours. No colonies formed on plates streaked out at 10 or 12 hours. The 14 and 16 hour plates again yielded very small, slow growing colonies. These colonies also formed zygotic asci indicating that they were haploid prototrophs, as found previously, rather than diploids.

Crosses between *S. pombe* strains and malate dependent strains derived from *S. malidevorans* mutant 11 have proved difficult to analyse. That is, very few asci were seen microscopically when the two parents were mixed on SPAM, often only prototrophic progeny were obtained, the spore viability is very low and expected segregation patterns were not obtained. Crosses with *S. pombe* malate dependent mutants did not yield data on spore viability or gene segregation ratios. However, there were problems with viability of uracil requiring malate dependent mutants on MEA plates, the lack of zygotes seen on MEA plates and the failure to detect any obvious diploids on minimal plates from crosses involving *S. pombe* malate dependent mutants. This strongly suggests that the malate dependent mutation, rather than species incompatibility, was the cause of low mating frequency and the lack of clear genetic data from crosses.

5.3. Complementation tests between malate dependent mutants of *S. pombe* and *S. malidevorans.*

Results of crosses between various malate dependent mutants of *S. malidevorans* suggested that there may be more than one complementation group (Rodrigeuz. personal communication; Hansen personal communication). WT 6, WT 7 and WT 8 were crossed with a *S. pombe*/*S. malidevorans* derivative of mutant 11, XL1 b (*ade 6*, mig$^-$), and with each other in order to analyse their complementation patterns. The results (Table 17) can only be considered tentative because the same problems were encountered in these crosses as those seen in crosses of the malate dependent *S. pombe* strains with auxotrophic *S. pombe*. When apparently diploid cells were forced to sporulate by streaking on SPAM, the asci appeared to be zygotic. On dissection of at least 20 asci from any cross that yielded growth on minimal plates, only prototrophic spores were found. The spore viability of these crosses was at least 80%. Reversion to malate independence was ruled out by failure of any strain to grow on minimal plates (Table 16). The data suggests that mutant 11 may be mutant in a different gene than the *S. pombe* mutants.

Table 17: Complementation analysis of malate dependent mutants of *S. pombe* and mutant 11.

Strains	WT 6	WT 7	WT 8	11
WT 6	–	–	–	+
WT 7		–	–	+
WT 8			–	+
11				–

+ = diploids were formed

– = diploids were not formed and no reversion occurred

6. Ploidy of malate dependent strains

Due to the non-Mendelian segregation patterns obtained in matings involving malate dependent strains, attempts were made to establish the ploidy of *S. malidevorans* 442 and malate dependent mutants of both *S. malidevorans* 442 and *S. pombe*. *S. pombe* is predominantly haploid (Gutz *et al.* 1974). The DNA content work of Murray (1989) suggested that *S. malidevorans* 442 may be haploid, as it had the same DNA content as haploid *S. pombe*. This work also showed that mutant 11 had a DNA content 1.44 times that of the wild-type. Magdala red plates were used to try to confirm these results and to extend the analysis to other strains.

This study confirmed previous observations (Kohli *et al.* 1977), that heterothallic *S. pombe* haploids and diploids stained light pink and dark pink respectively when streaked onto MR plates. Homothallic *S. pombe* strains always stained lighter pink than diploids and darker than haploids, after 3-4 days. Microscopic examination of the homothallic colonies over time showed that darker staining colonies had formed zygotes.

S. malidevorans 442 was indistinguishable from *S. pombe* homothallic strains on these plates, which were an intermediate colour between the light pink colour of haploids and the dark pink colour of diploids (Table 18). Another study showed that the DNA content of *S. malidevorans* 442 was equivalent to the DNA content of haploid *S. pombe* strains (Murray 1989). Other evidence that

Table 18: Colour of *Schizosaccharomyces* strains on magdala red plates.

Schizosaccharomyces strains	Light Pink	Intermediate Pink	Dark Pink
S. pombe strains heterothallic/haploid	+		
442 heterothallic	+		
Malate independent *S. pombe/S. malidevorans* hybrids: heterothallic	+		
S. pombe strains homothallic/haploid		+	
S. malidevorans 442 homothallic		+	
Class 1 revertant (spo+)		+	
Malate independent *S. pombe/S. malidevorans* hybrids: homothallic		+	
S. pombe diploids			+
S. pombe malate dependent mutants			+
Mutant 11			+
Class 2 revertant (spo-)			+
Malate dependent *S. pombe/S. malidevorans* hybrids (spo-)			+

S. malidevorans 442 is predominantly haploid was that zygotic asci were produced when these cells mated. Azygotic asci, produced when diploids undergo meiosis (Gutz *et al.* 1974) were not detected. To investigate the possibility that different liquid media may affect the stage of the life cycle seen on the MR plates, *S. malidevorans* 442 was cultured in different media, i.e. YEA, MM and YMM, until either exponential or stationary phase and then streaked out on MR plates. Conjugation and sporulation was never seen in any liquid culture in this study. It was assumed that whatever stage the cells were in upon inoculation into liquid, that they remained predominantly in that stage. Varying the media or stage of growth had no effect on the colour of *S. malidevorans* 442 on MR plates.

Colonies of mutant 11 stained dark pink on MR plates. Twenty two *S. pombe*/*S. malidevorans* hybrids from the cross between *S. pombe* 122 and malate dependent hybrid XL1 b were streaked out on MR plates. All malate dependent hybrids were dark pink, and all the malate independent progeny were light pink, if heterothallic, or an intermediate colour between light and dark pink, if homothallic (Table 18). If malate dependent strains were diploid and unable to undergo meiosis it would explain why mating was rare with malate dependent and haploid strains, why low spore viabilities were obtained and why segregation patterns were complex. Magdala red is a vital stain and is known to stain UV sensitive, temperature sensitive, drug resistant and some auxotrophic mutants dark pink. Thus, these results could indicate that malate dependent mutants are diploid or that a mutation in mutant 11 results in a lower viability than the wild-type. The DNA content of mutant 11 was found to be 1.44 times the DNA content of *S. malidevorans* 442 (Murray1989), supporting the results obtained using MR plates.

Class 1 revertants are malate independent and have regained the ability to sporulate, were indistinguishable from *S. malidevorans* 442 on MR plates. Class 2 revertants are malate independent but are unable to sporulate, were dark pink on MR plates. These results indicated that malate dependence did not affect colour on MR plates, and suggested a correlation between dark colour on MR plates and an inability to sporulate.

Malate dependent mutants of *S. pombe* were dark pink on MR plates, similar to the malate dependent *S. malidevorans* mutants (Table 18).

The results obtained in this study confirm the findings of Murray (1989), suggesting that mutant 11 is aneuploid, possibly due to chromosomal rearrangement. If malate dependence is due to chromosomal rearrangement, obtaining mig^{+} clones by transformation may not be possible.

7. Transverse Alternating Field Electrophoresis (TAFE)

The difficulty of genetic analysis of crosses between mutant 11 *S .pombe* strains, and the increased DNA content of mutant 11, suggested the possibility of chromosomal rearrangement or aneuploidy. Pulsed field gel electrophoresis was used to examine these possibilities. *S. pombe* has three chromosomes with

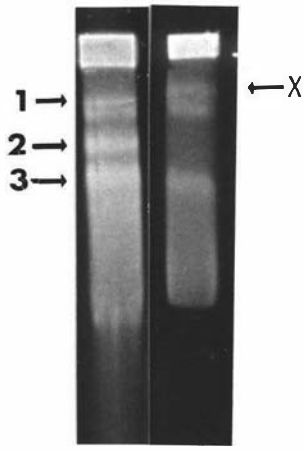
Plate 5: Chromosome patterns of *S. malidevorans* strains, obtained by TAFE.

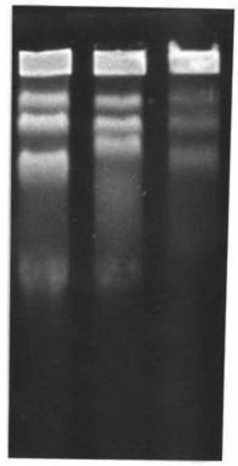
Lane 1, *S. malidevorans* 442;
Lane 2, mutant 11;
Lane 3 and lane 4, two adenine mutants of *S. malidevorans* 442.
Lane 5, S. malidevorans 442.

Plate 6: Chromosome patterns of *S. malidevorans* and hybrid strains, obtained by TAFE.

Lane 1 and lane 2, two class 1 revertants;
Lane 3, *S. malidevorans* 442;
Lane 4, mutant 11;
Lane 5 and lane 6, malate dependent *S. malidevorans/S. pombe* hybrids, XW1 5, and XW1 15;
Lane 7 and lane 8, two class 2 revertants;
Lane 9, malate independent hybid XW1 2;

Lane 10, mutant 11;
Lane 11, malate independent hybrid XW1 6.


1
2
3
X



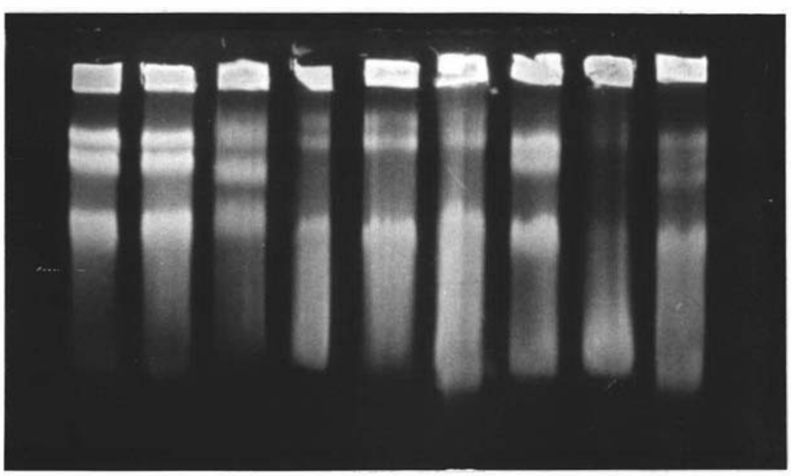
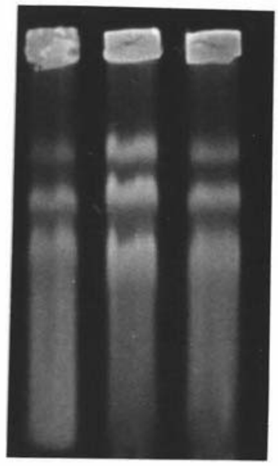


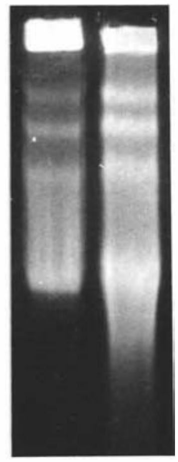
Plate 7: **Chromosome patterns of *S. pombe* strains, obtained by TAFE.**

Lane 1, malate dependent mutant WT 6;
Lane 2, *S. pombe* WT 4;
Lane 3, a class 1 revertant;

Lane 4, *S. pombe* WT 4;
Lane 5 and lane 6, two adenine mutants of WT 4;

Lane 7, *S. pombe* WT 5;
Lane 8, malate dependent mutant, WT 8.





a karyotype similar to mutant 11. The other had a karyotype similar to the class 1 revertants (Plate 6).

Two malate dependent and two malate independent progeny of an *S. pombe* and mutant 11 cross were karyotyped. The malate dependent progeny had an identical pattern to that of mutant 11 (Plate 6). The malate independent progeny, however, were not identical to each other nor to *S. malidevorans* 442 or *S. pombe* strains. XW1 6 appeared to have two chromosomes of equal size (Plate 6). XW1 2 appeared to have four chromosomes (Plate 6).

S. pombe malate dependent mutants were karyotyped and had a different pattern from mutant 11. Two separate cell preparations showed that band 2 of WT 6 appeared to have shifted slightly from the *S. pombe* WT 4 position (Plate 7), but was still located between bands 1 and 3, like that of mutant 11. Band 3 of WT 6 also appeared to have shifted to a position suggesting it was larger than the wild-type band 3 (Plate 7). An apparently full revertant of WT 6 had a karyotype indistinguishable from the mutant WT 6 pattern (Plate 7). Two adenine mutants of *S. pombe* WT 4 had very similar karyotypes to that of *S. pombe* WT 4 (Plate 7). The karyotype of another malate dependent mutant of *S. pombe*, WT 8, appeared to differ only slightly, if at all, from the wild-type *S. pombe* WT 5 pattern (Plate 7). The apparent alterations were slight increases in the sizes of bands 2 and 3. A gel that was run for 144 hours failed to make the difference between wild-type and mutant any clearer.

8. Enzyme Assays

This study confirmed earlier work (Rodriguez and Thornton 1989) that mutant 11 uses more malate and less glucose than the wild-type, *S. malidevorans* 442 (Figures 1 and 2). A malate dependent mutant of *S. pombe* WT 4, WT 6, also uses more malate and less glucose than *S. pombe* WT 4 (Figures 3 and 4). Altered malate and glucose metabolism could be due to alterations in the enzymes involved in catabolism of either glucose or malate. Assays of enzymes that may by involved in this phenotype were done.

8.1. Malic enzyme

Malic enzyme activities of *S. malidevorans* 442 and mutant 11 were assayed. The specific activity of malic enzyme in mutant 11 was 0.4 μmol/min/mg protein, almost 10-fold higher than the specific activity of wild-type at 0.045 μmol/min/mg protein (Table 19).

Two representatives from each of the two revertant classes were also assayed for malic enzyme. The class 1 revertants had malic enzyme activity approximately double that of *S. malidevorans* 442, and the class 2 revertants have malic enzyme activity very similar to mutant 11 (Table 19).

In order to test whether increased malic enzyme activity was specific to malate dependent hybrids two malate dependent hybrids, XW1 5 and XW1 15, and two malate independent hybrids,

Table 19: Specific activities of malic enzyme of *Schizosaccharomyces* strains.

Schizosaccharomyces strains	Specific activity (μmol product/min/mg protein)
S. malidevorans 422 (mig$^+$)	0.045
Mutant 11 (mig$^-$)	0.40
Class 1 revertant (mig$^+$)	0.11
Class 2 revertant (mig$^+$)	0.41
XW1 5, XW1 15 (mig$^-$)	0.42
XW1 2 (mig$^+$)	0.067
XW1 6 (mig$^+$)	0.062
S. pombe WT 4 (mig$^+$)	0.10
WT 6 (mig$^-$)	0.11

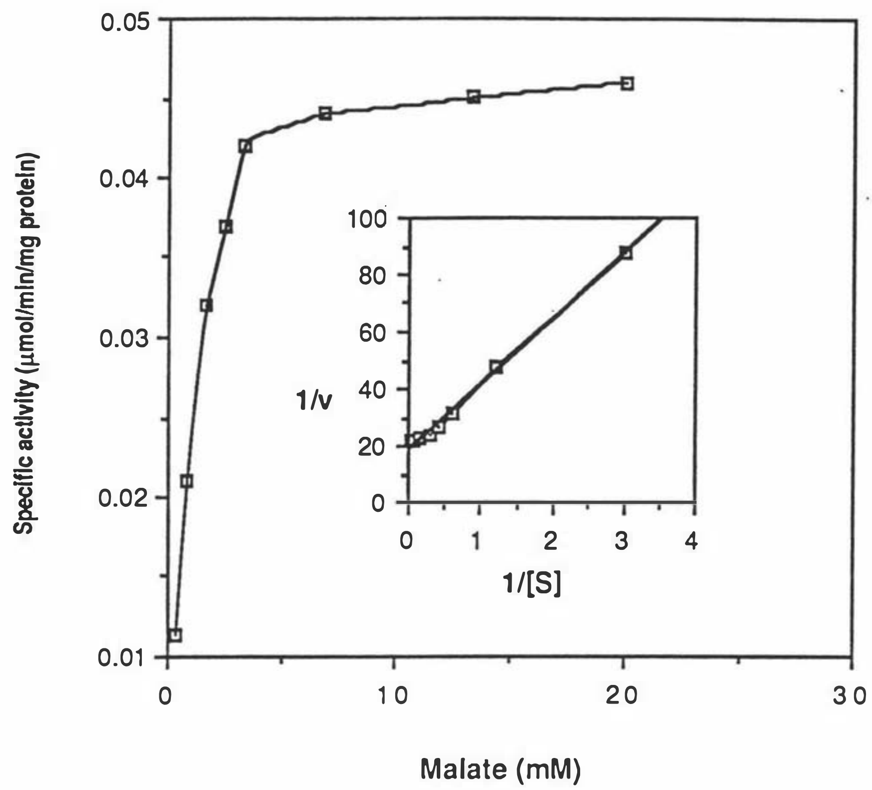
XW1 2 and XW1 6 *S. pombe/S. malidevorans* hybrids from the cross *S. pombe/S. malidevorans* hybrid, XL1 b (mig$^-$, *ade 6-704*) and *S. pombe* 122 h^- (*leu 1, his 2*) were assayed for malic enzyme activity. Both malate dependent hybrids had specific activities very similar to mutant 11. The two malate independent hybrids had low specific activities, although not as low as *S. malidevorans* 442.

Since the specific activities of *S. malidevorans* 442 and mutant 11 were found to differ greatly, kinetics of the malic enzyme using cell-free extracts of *S. malidevorans* 442 and mutant 11 were done (Figures 5-8). The K_m values for malic acid of the malic enzyme of *S. malidevorans* 442 and mutant 11 were 1.203 mM and 1.413 mM, respectively. The K_m values for NAD of the malic enzyme of *S. malidevorans* 442 and mutant 11 were 0.334 mM 0.339 mM, respectively. These data indicate that the affinities of malic enzyme for malate and NAD in mutant 11 have not been altered.

The malic enzyme activities of *S. pombe* (WT 4) and malate dependent mutant WT 6 were not significantly different, unlike *S. malidevorans* 442 and mutant 11. The specific activities of the *S. pombe* strains were approximately twice the activity of *S. malidevorans* 442 (Table 19). The kinetics of malic enzymes in *S. pombe* were not done because no difference in activity was observed.

Figure 5: **Saturation and Lineweaver-Burke plots of the malic enzyme of *S. malidevorans* 442, varying concentrations of malic acid.**

Figure 6: **Saturation and Lineweaver-Burke plots of the malic enzyme of *S. malidevorans* mutant 11, varying concentrations of malic acid.**


0.05
0.04
0.03
0.02
0.01
Specific activity (µmol/min/mg protein)
0
10
20
30
Malate (mM)
100
80
60
40
20
0
1/v
0
1
2
3
4
1/[S]

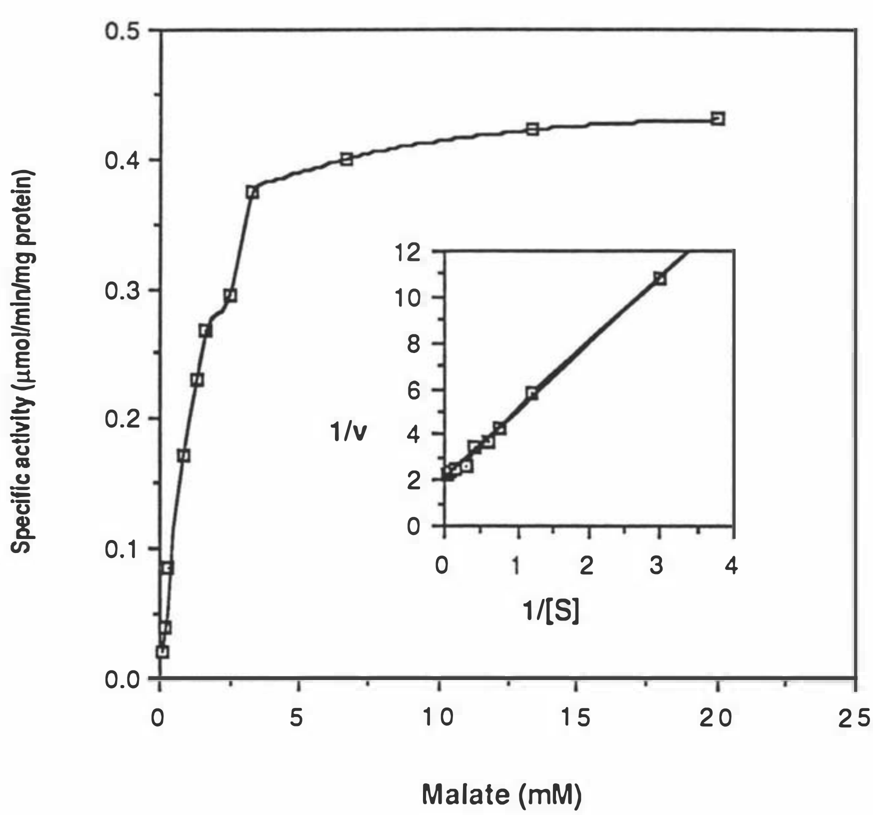
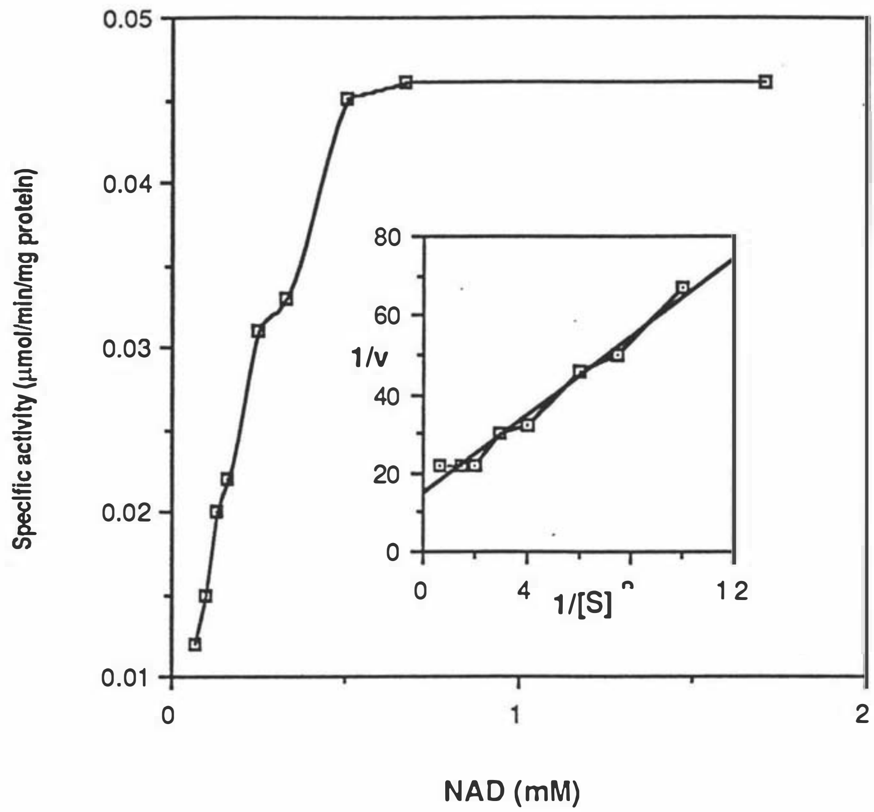
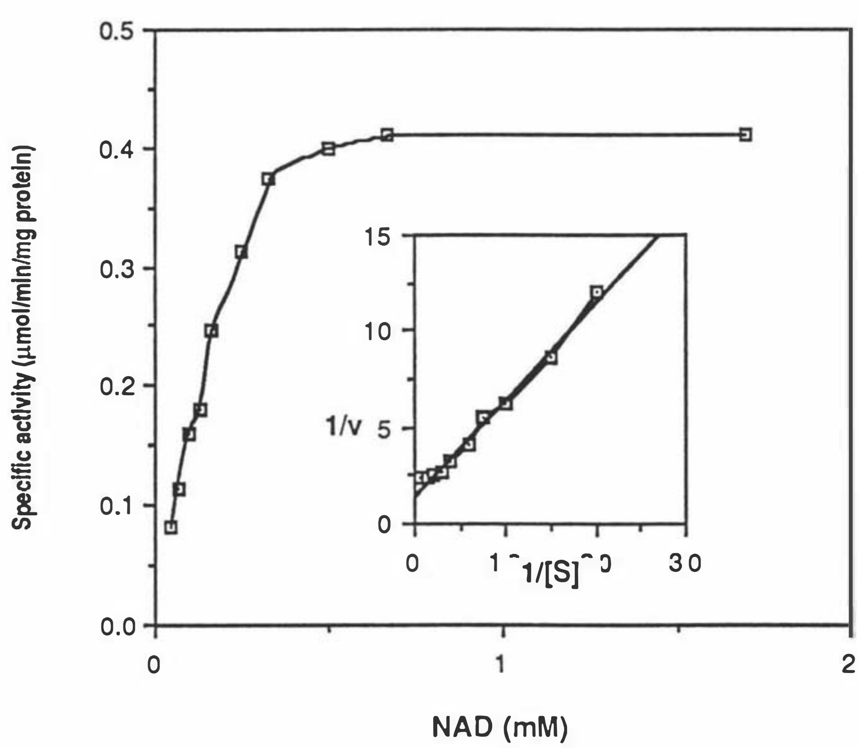

0.5
0.4
0.3
0.2
0.1
0.0
Specific activity (µmol/min/mg protein)
0
5
10
15
20
25
Malate (mM)
12
10
8
6
4
2
0
1/v
0
1
2
3
4
1/[S]

Figure 7: **Saturation and Lineweaver-Burke plots of the malic enzyme of *S. malidevorans* 442, varying concentrations of NAD.**

Figure 8: **Saturation and Lineweaver-Burke plots of the malic enzyme of *S. malidevorans* mutant 11, varying concentrations of NAD.**


0.05
0.04
0.03
0.02
0.01
Specific activity (µmol/min/mg protein)
0
1
2
NAD (mM)
80
60
40
20
0
1/v
0
4
12
1/[S]


0.5
0.4
0.3
0.2
0.1
0.0
Specific activity (µmol/min/mg protein)
0
1
2
NAD (mM)
15
10
5
0
1/v
0
30
1/[S]

8.2. Malate dehydrogenase (MDH)

MDH activity was assayed in some of the same strains as were tested for malic enzyme activity. There were no significant differences in MDH activity among these strains (Table 20). The glucose concentration of a medium is known to affect the specific activity of MDH in *S. pombe.* At glucose concentrations below 1 g/l, malate dehydrogenase is derepressed and the activity is increased 20-fold (Flury *et al.* 1974). YMM media, after growth of the cells for MDH assays, was always found to contain more than 2g/l glucose, estimated by Diastik. These data indicate that the malate dependent mutants' ability to utilise more malate than *S. malidevorans* 442 and *S. pombe* is not due to derepressed malate dehydrogenase at high glucose concentrations.

8.3. Hexose-ATP-kinase

Hexokinase assays were done on cell-free extracts of both *S. malidevorans* 442 and mutant 11. The assays showed no significant difference in specific activity (Table 21).

8.4 Thermal inactivation of malic enzyme

The activities of the malic enzyme from *S. malidevorans* 442 and mutant 11 were significantly different. To test for a difference in protein structure between the mutant and wild-type enzyme, thermal inactivation of malic enzyme from crude extracts of *S. malidevorans* 442 and mutant 11 was done. The thermal

Table 20: Specific activities of malate dehydrogenase of *Schizosaccharomyces* strains.

Strains	Specific activity (μmol product/min/mg protein)
S. malidevorans 422 (mig^+)	0.08
Mutant 11 (mig^-)	0.071
Class 1 revertants (mig^+)	0.072
Class 2 revertants (mig^+)	0.062
S. pombe WT 4 (mig^+)	0.131
WT 6 (mig^-)	0.112

Table 21: Specific activities of ATP - hexokinase for *S. malidevorans* strains.

Strains	Specific activity (µmol product/min/mg protein)
S. malidevorans 442	0.29
Mutant 11	0.27

inactivation curve of the malic enzyme of mutant 11, unlike *S. malidevorans* 442, was biphasic (Figure 9). This result suggests the presence of two types of malic enzyme. One is a heat stable enzyme with an estimated specific activity equivalent to the specific activity of the wild-type. The other is a heat sensitive enzyme representing approximately 90 percent of the malic enzyme activity of mutant 11 (Figure 9).

The heat inactivation of malic enzyme from both classes of revertant is shown in Figure 9. Revertant class 2 behaved similarly, in heat inactivation experiments, to mutant 11. Revertant class1 also appeared to have two types of enzyme. The stable enzyme of class 1 represents approximately 50 percent of the total malic enzyme activity, the activity of which is very similar to that of *S. malidevorans* 442. Neither class of revertant had an identical pattern of inactivation to that of *S. malidevorans* 442.

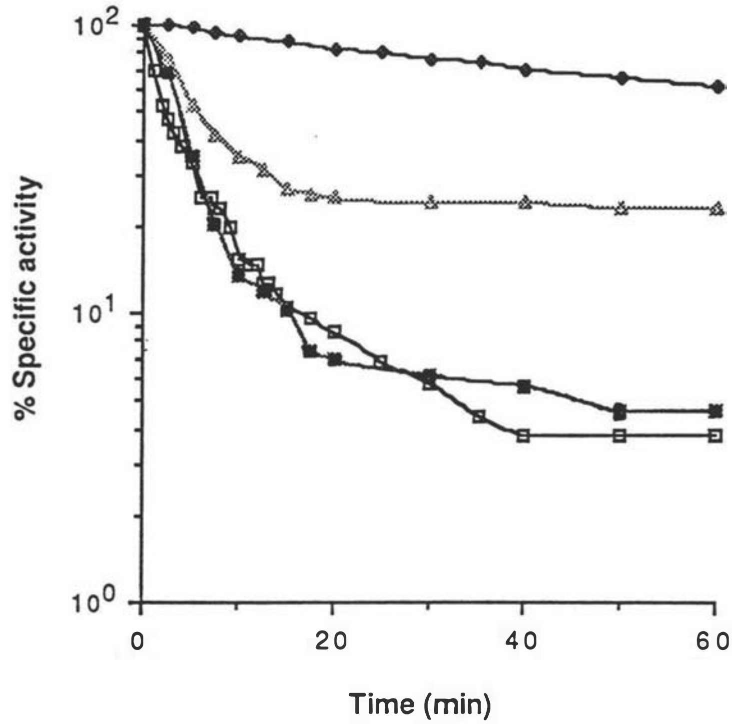
Heat inactivation studies of the malic enzymes of *S. pombe* WT 4 and mutant WT 6 confirmed that malic enzyme in WT 6 is not altered from wild-type (Figure 10). There seems to be a small amount of biphasic heat inactivation in *S. pombe* and WT 6 compared with the heat inactivation of *S. malidevorans* 442 (Figure 10).

9. Uptake of substrates by *Schizosaccharomyces* strains

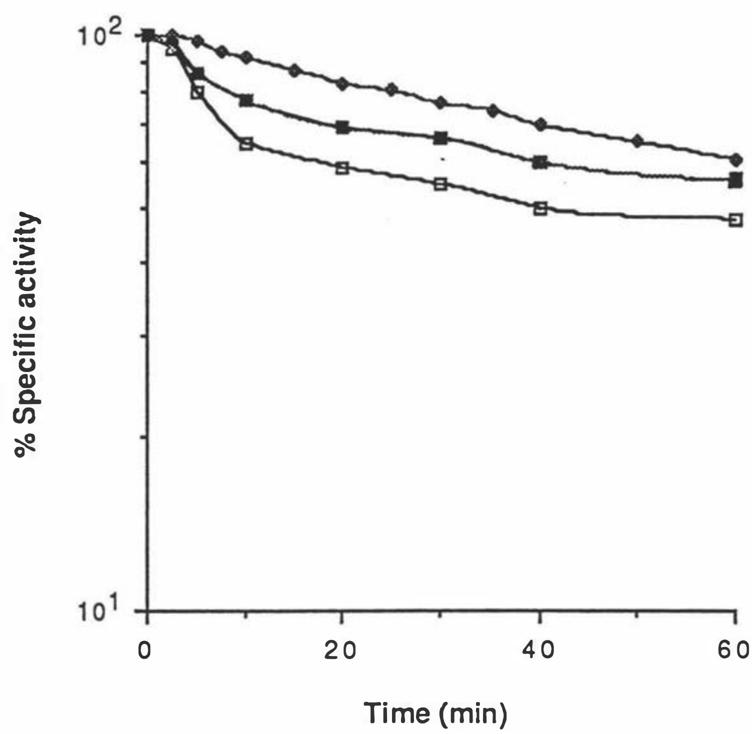
Alterations in metabolism of glucose and malate by malate dependent mutants may be due to alterations in transport of

Figure 9: Heat inactivation of malic enzymes from *S. malidevorans* 442, ◆, mutant 11, □, a class 1 revertant, Δ, and a class 2 revertant, ■.

Figure 10: Heat inactivation of malic enzymes from *S. malidevorans* 442, ◆, *S. pombe* WT 4, ■, and the malate dependent mutant, WT 6, □.



% Specific activity
10^2
10^1
10^0
0
20
40
60
Time (min)



% Specific activity
10^2
10^1
0
20
40
60
Time (min)

malate and glucose. This possibility was investigated. Since uptake assays were done over a period of thirty minutes the results reflect both uptake and metabolism.

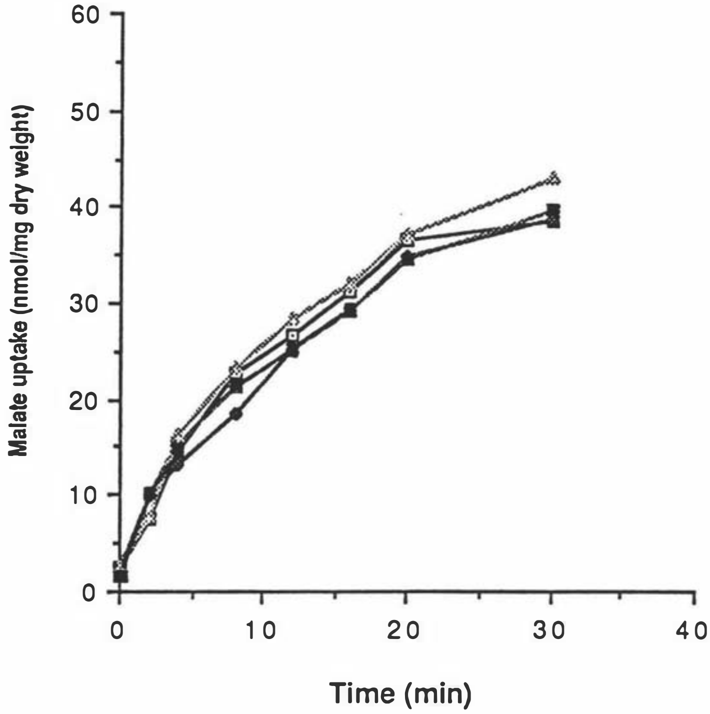
9.1. Malate uptake

Uptake of ^{14}C malate, at pH 3.5 by *Schizosaccharomyces* strains was investigated. Uptake of malate was found to be very similar in *S. malidevorans* 442, mutant 11 and in representatives of both classes of revertants (Figure 11). Since mutant 11 requires both malate and glucose for growth, uptake studies were also done with both malate and glucose to check how the uptake of one substrate was affected by the other. When non-labelled glucose was added to the malate uptake assays mutant 11 and was distinguished from the wild-type. Malate uptake by *S. malidevorans* 442 was markedly reduced in the presence of glucose, whereas malate uptake by mutant 11 was not (Figure 12). Inhibition of malate uptake by glucose was evident in both classes of revertants although to a lesser extent than for *S. malidevorans* 442 (Figure 12).

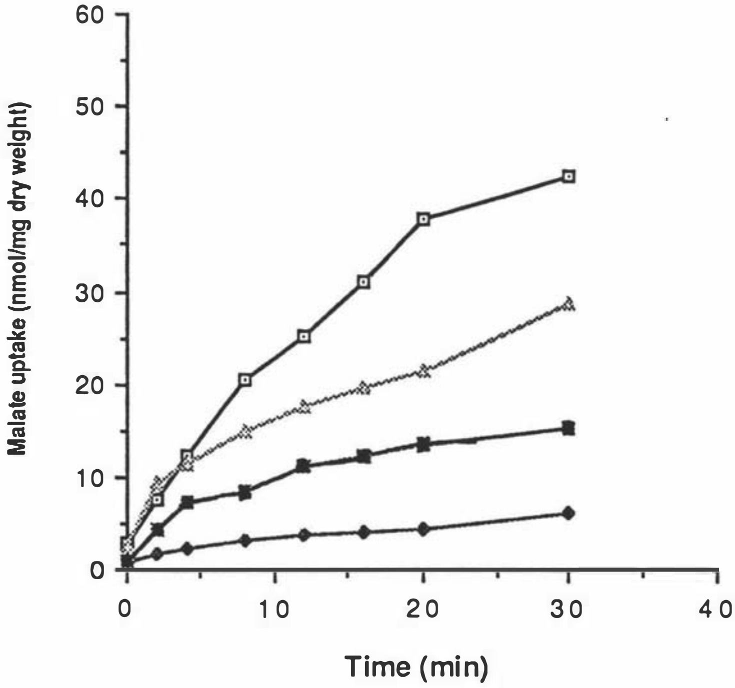
Malate uptake was similar in *S. pombe* WT 4, malate dependent mutant WT 6 and a revertant of WT 6 (Figure 13). Malate uptake in *S. pombe* WT 4 is reduced in the presence of glucose (Figure 14), although the degree of inhibition of malate uptake by glucose is less than in *S. malidevorans* 442 (Figure 12). Inhibition of malate uptake by glucose is not evident in the malate dependent mutant WT 6, similar to mutant 11, and is partially regained in a revertant (Figure 14).

Figure 11: Malate uptake by *S. malidevorans* 442, ◆, mutant 11, □, class 1 revertant, ■ , and class 2 revertant, Δ.

Figure 12: Malate uptake, in the presence of glucose, by *S. malidevorans* 442, ◆, mutant 11, □ , class 1 revertant, ■ , and class 2 revertant, Δ.



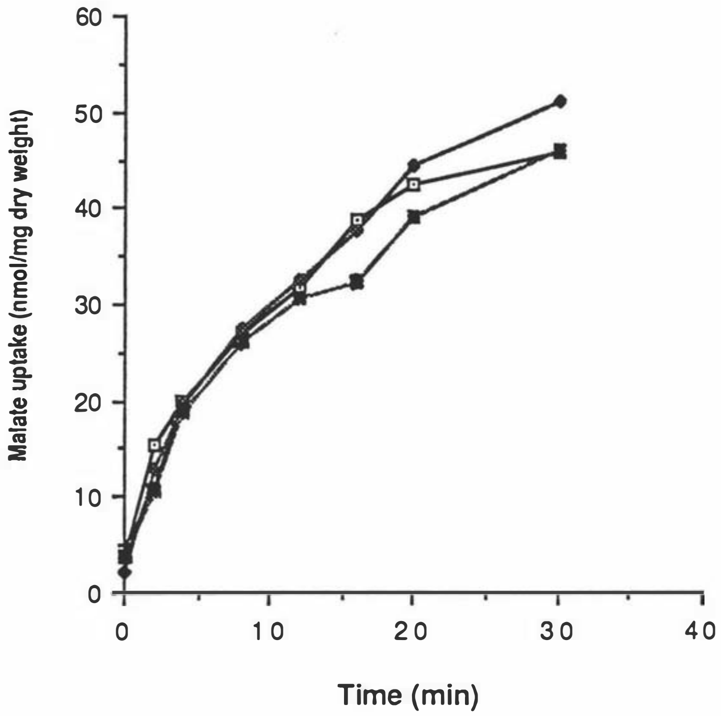
Malate uptake (nmol/mg dry weight)
60
50
40
30
20
10
0
0
10
20
30
40
Time (min)



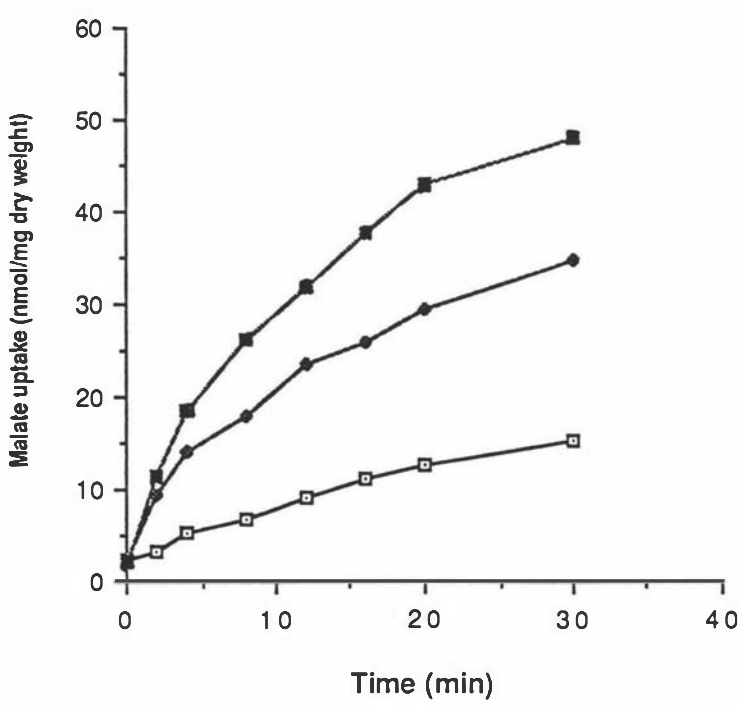
Malate uptake (nmol/mg dry weight)
60
50
40
30
20
10
0
0
10
20
30
40
Time (min)

Figure 13: **Malate uptake by *S. pombe* WT 4, □, malate dependent mutant WT 6, ■, and a class 1 revertant, ◆.**

Figure 14: **Malate uptake, in the presence of glucose, by *S. pombe* WT 4, □, mutant WT 6, ■, and a class 1 revertant, ◆.**



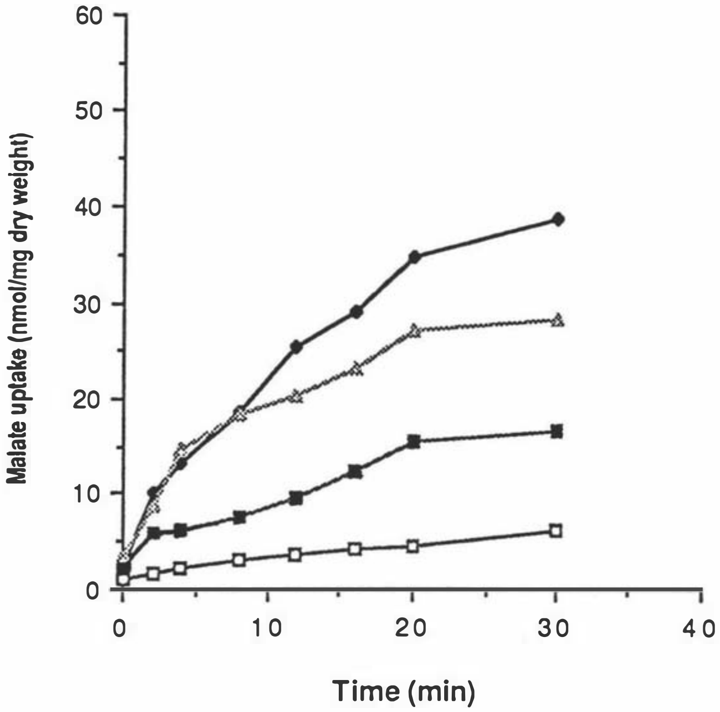
Malate uptake (nmol/mg dry weight)
60
50
40
30
20
10
0
0
10
20
30
40
Time (min)



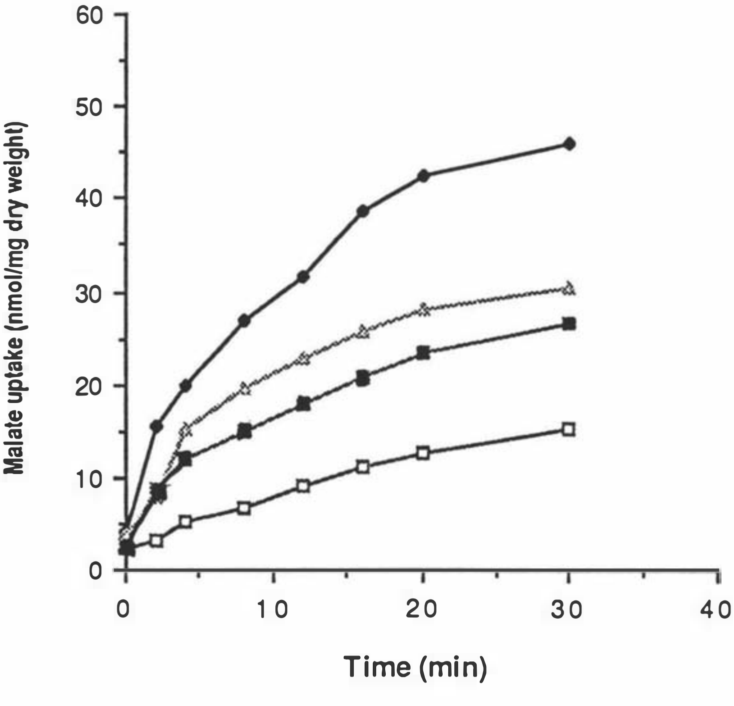
Malate uptake (nmol/mg dry weight)
60
50
40
30
20
10
0
0
10
20
30
40
Time (min)

Figure 15: Uptake, by *S. malidevorans* 442, of malate, ◆, malate in the presence of glucose, □, and malate in the presence of succinate, ■. Uptake of malate in the presence of succinate by mutant 11, Δ.

Figure 16: Malate uptake, by *S. pombe* WT 4, ◆, malate in the presence of glucose, □, in the presence of succinate, ■, and by WT 6 in the presence of succinate, Δ.



Malate uptake (nmol/mg dry weight)
60
50
40
30
20
10
0
0
10
20
30
40
Time (min)



Malate uptake (nmol/mg dry weight)
60
50
40
30
20
10
0
0
10
20
30
40
Time (min)

The effect of another 4-carbon TCA-cycle intermediate, succinic acid, on malate uptake was examined. Malate uptake in *S. malidevorans* 442 was inhibited by succinate (Figure 15). Despite complete loss of glucose inhibition of malate uptake by mutant 11 (Figure 12), succinate did inhibit malate uptake, although not to the same degree as it did in *S. malidevorans* 442 (Figure 15).

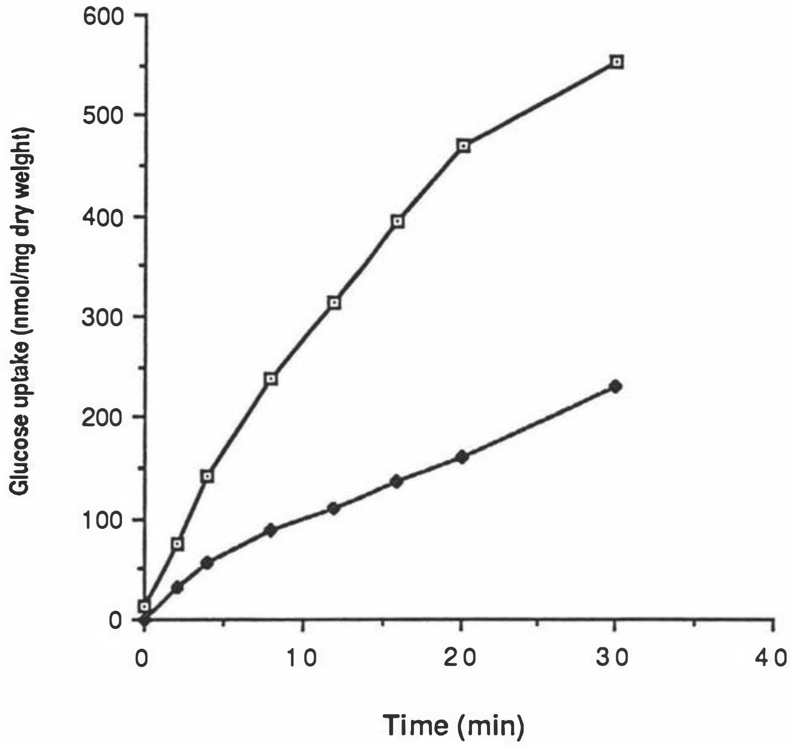
Malate uptake by *S. pombe* WT 4 was inhibited by succinate (Figure 16). Although WT 6 has lost the glucose inhibition of malate uptake (Figure 14), succinate still inhibited malate uptake (Figure 16). In fact, malate uptake by WT 6 is as strongly inhibited by succinate as it is in *S. pombe* WT 4 (Figure 16).

Malate uptake by *S. malidevorans* 442 and *S. pombe* WT 4 was similar. The effects of glucose and succinate on malate uptake by *S. malidevorans* 442 and *S. pombe* WT 4 was very similar. Inhibition of malate uptake by succinate but not by glucose are features of both mutant 11 and the *S. pombe* malate dependent mutant WT 6.

9.2. Glucose uptake

Glucose uptake by *S. malidevorans* 442 and mutant 11 was measured at two pH values, pH 7.0, more commonly used for glucose uptake experiments, and pH 3.5, at which malate uptake experiments were conducted. Mutant 11 does not grow in media at pH values higher than 5.2, and most media used to grow

Figure 17: Glucose uptake of *S. malidevorans* 442 at pH 7.0, ◆, and at pH 3.5, □.



Glucose uptake (nmol/mg dry weight)
600
500
400
300
200
100
0
0
10
20
30
40
Time (min)

Schizosaccharomyces in this study were in the range pH 3.0 to pH 4.5. It was found that glucose uptake by *S. malidevorans* 442 was much higher at pH 3.5 than at pH 7.0 (Figure 17). All subsequent uptake experiments were done at pH 3.5.

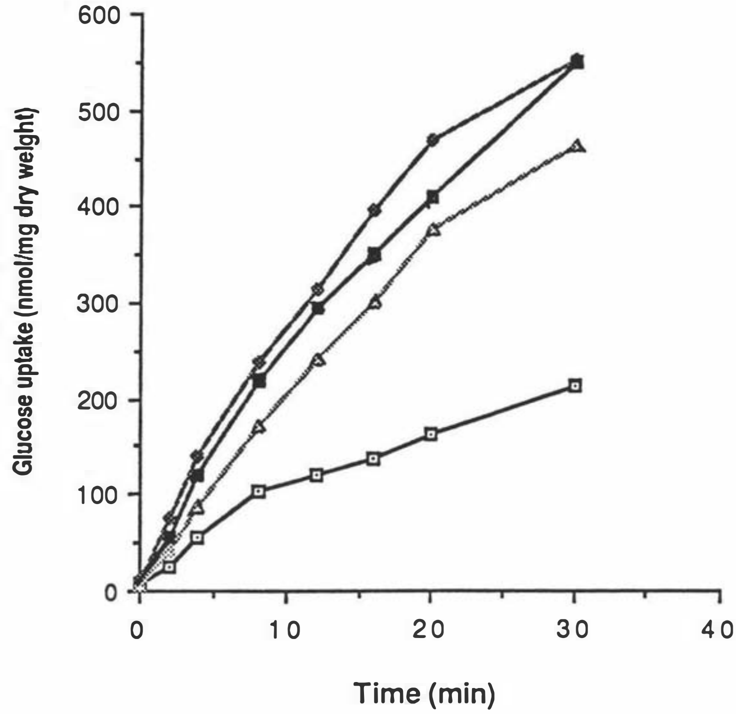
Glucose uptake was measured in malate dependent mutants of *S. malidevorans* and *S. pombe*. Unlike malate uptake, glucose uptake was greatly affected in the malate dependent strains. Glucose uptake by mutant 11 was 38%, at 30 minutes, of that taken up by *S. malidevorans* 442 (Figure 18). Both classes of revertants had ability to take up more glucose than mutant 11. The class 1 revertant regained the ability to take up glucose as well as *S. malidevorans* 442, and the class 2 revertant transported glucose almost as much (82%) as *S. malidevorans* 442 (Figure 18).

Addition of non-labelled malate to glucose uptake assays further distinguished the *S. malidevorans* strains from their respective wild-type strains and from each other.

Glucose uptake by *S. malidevorans* 442 and by the class 1 revertant of mutant 11 in the presence of cold malate was inhibited (Figure 19). Glucose uptake by *S. malidevorans* 442 was inhibited by 44% and the class 1 revertant was inhibited by 24% (Figure 19). Glucose uptake by mutant 11 and the class 2 revertant were unaffected by malate (Figure 19).

Figure 18: Glucose uptake by *S. malidevorans* 442, ◆, by mutant 11, □ , a class 1 revertant, ■ , and a class 2 revertant, Δ.

Figure 19: Glucose uptake, by *S. malidevorans* 442, ◆, by *S. malidevorans* 442 in the presence of malate, ■ , by mutant 11 in the presence of malate, □ , by a class 1 revertant in the presence of malate, ▲, and by a class 2 revertant in the presence of malate, Δ.


Glucose uptake (nmol/mg dry weight)
600
500
400
300
200
100
0
0
10
20
30
40
Time (min)

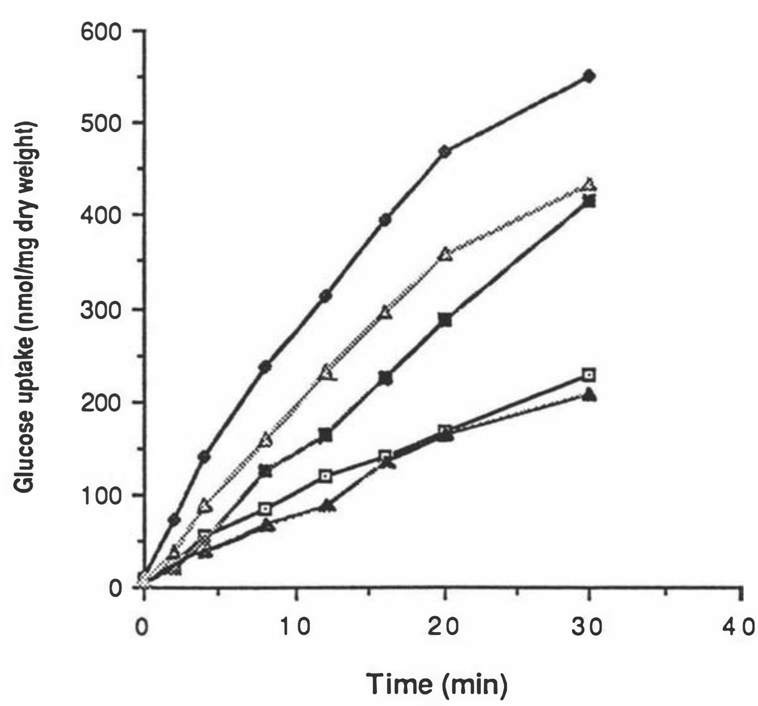
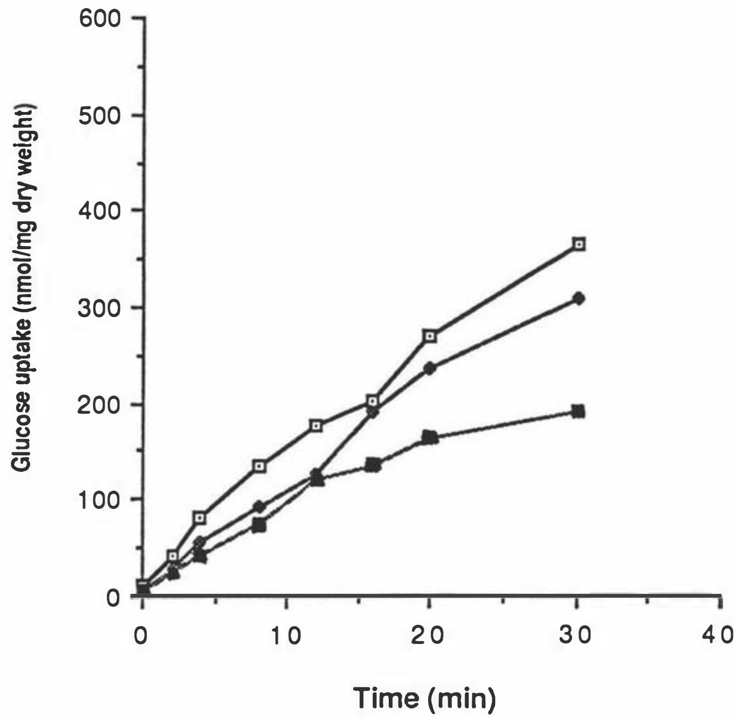
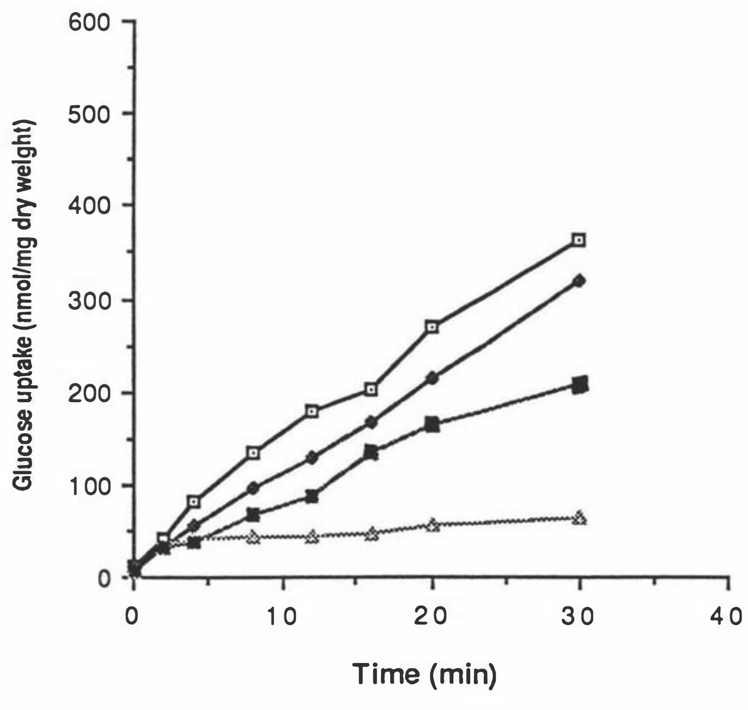

Glucose uptake (nmol/mg dry weight)
600
500
400
300
200
100
0
0
10
20
30
40
Time (min)

Figure 20: Glucose uptake by *S. pombe* WT 4, □, a class 1 revertant, ◆, and WT 6, ■.

Figure 21: Glucose uptake, by *S. pombe* WT 4, □, by *S. pombe* WT 4 in the presence of malate, ◆, by WT 6 in the presence of malate, Δ, and a class 1 revertant in the presence of malate, ■.


600
500
400
300
200
100
0
Glucose uptake (nmol/mg dry weight)
0
10
20
30
40
Time (min)


600
500
400
300
200
100
0
Glucose uptake (nmol/mg dry weight)
0
10
20
30
40
Time (min)

S. pombe WT 4 transported glucose twice as well as mutant WT 6 (Figure 20). The revertant was able to take up glucose almost as well (86%) as the wild-type (Figure 20).

Glucose uptake in the presence of malate by *S. pombe* WT 4 and by a class 1 revertant of WT 6, were both inhibited (Figure 21). Glucose uptake was inhibited by 13% in *S. pombe* WT 4 and to a greater extent (32%) in the revertant (Figure 21). Glucose uptake by WT 6 was markedly reduced by the addition of non-labelled malate to the glucose assay, i.e glucose uptake was only 32% of that transported by *S. pombe* WT 4 in the presence of cold malate (Figure 21).

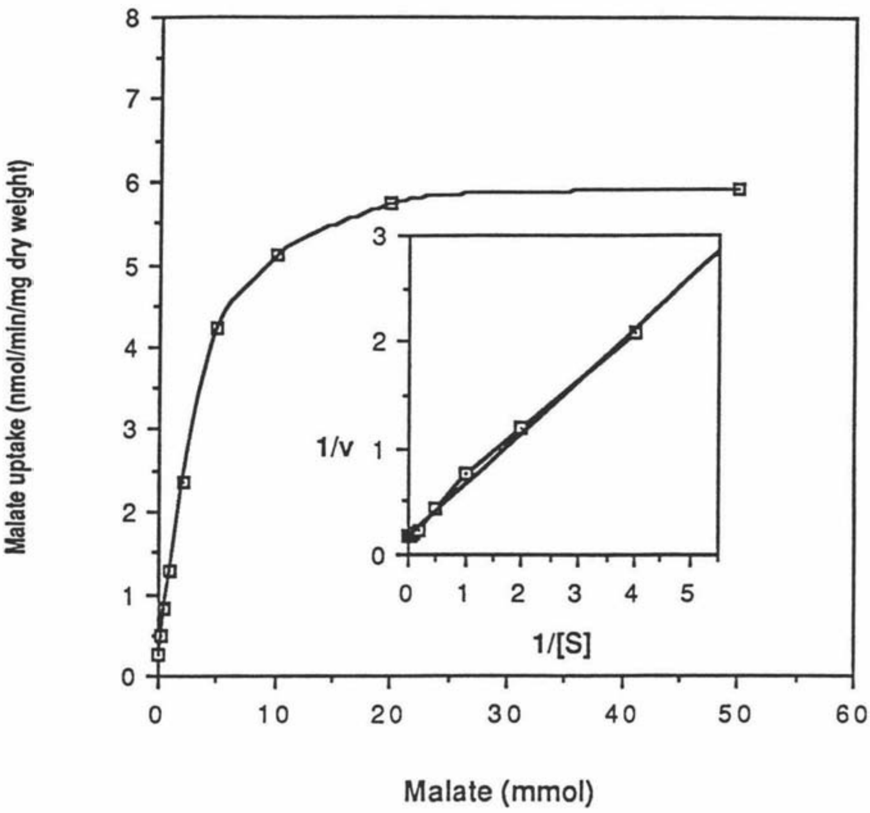
9.3. Kinetics of malate and glucose uptake

Utilisation of both malate and glucose, as measured by their disappearance from the medium, is altered in malate dependent mutants. The uptake of glucose in malate dependent mutants 11 and WT 6 is altered compared to wild-type transport. The K_m values for malate and glucose transport were estimated to investigate the changes in uptake of both these substrates in malate dependent mutants.

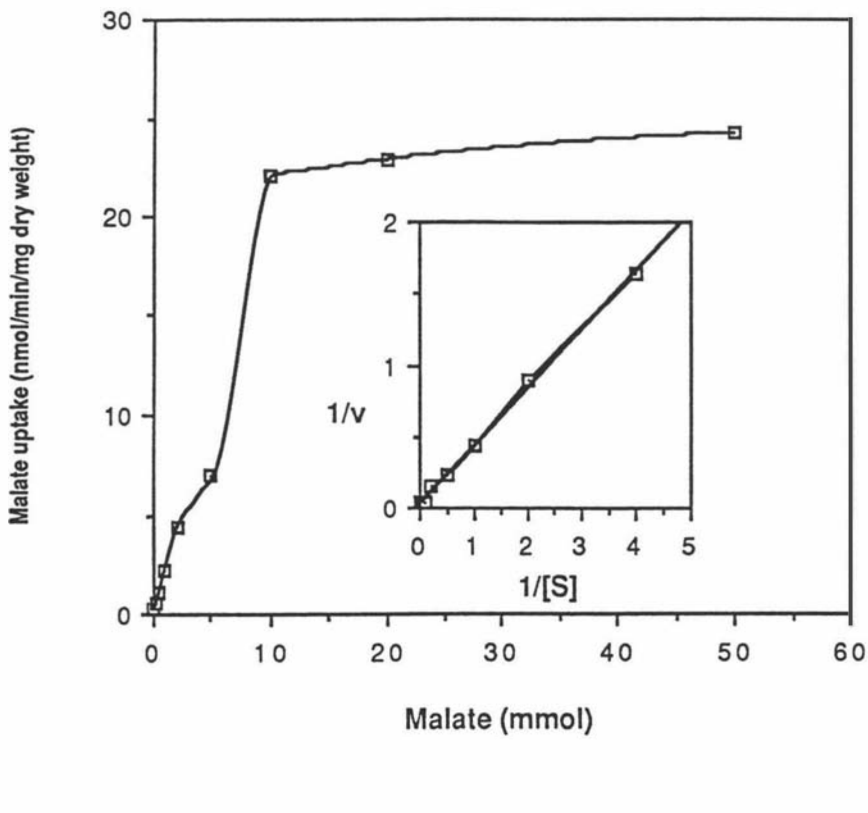
The estimated K_m for malate uptake of *S. malidevorans* 442 was 2.7 mM (Figure 22), which is very similar to the estimated K_m for malate uptake by *S. pombe*, 3.7 mM (Osothsilp and Subden, 1986). The V_{max} was 6.7 nmol/min/mg dry weight. The estimated K_m for malate transport by mutant 11 was 11.5 mM (Figure 23). The V_{max}

Figure 22: Saturation and Lineweaver-Burke plots of malate uptake in *S. malidevorans* 442

Figure 23: Saturation and Lineweaver-Burke plots of malate uptake in mutant 11



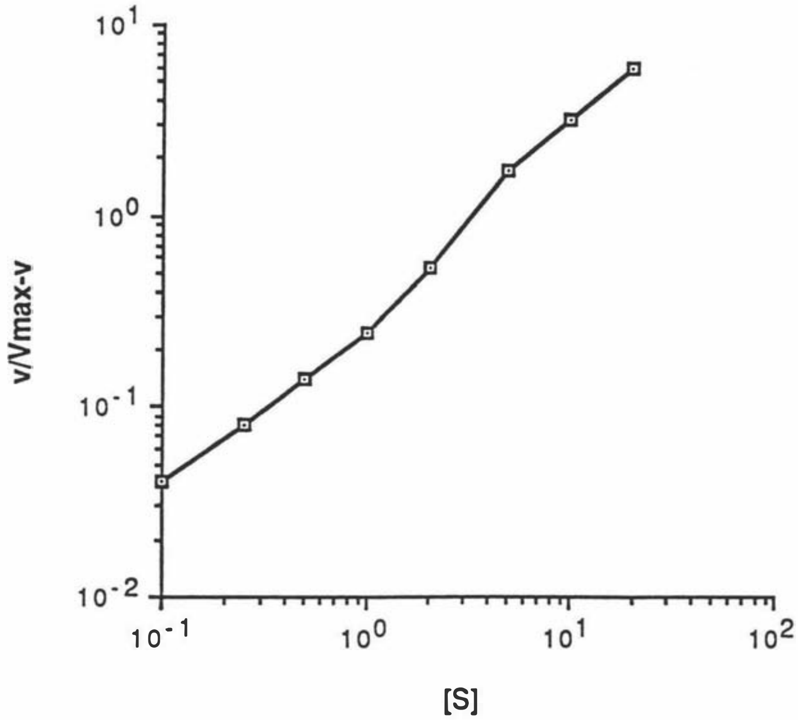
Malate uptake (nmol/min/mg dry weight)
Malate (mmol)
1/v
1/[S]



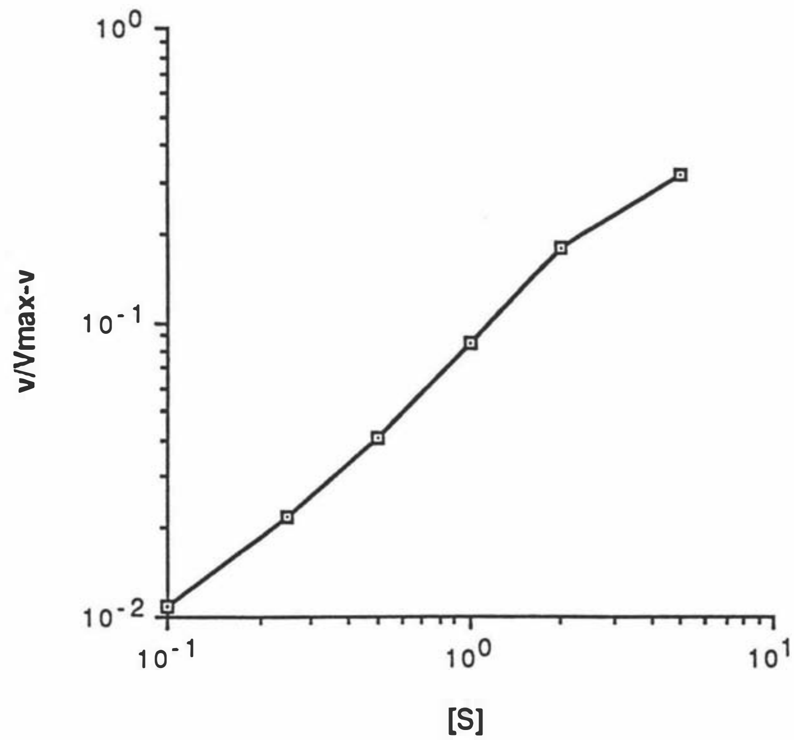
Malate uptake (nmol/min/mg dry weight)
Malate (mmol)
1/v
1/[S]

Figure 24: Hill plot of malate uptake by *S. malidevorans* 442.

Figure 25: Hill plot of malate uptake by mutant 11.



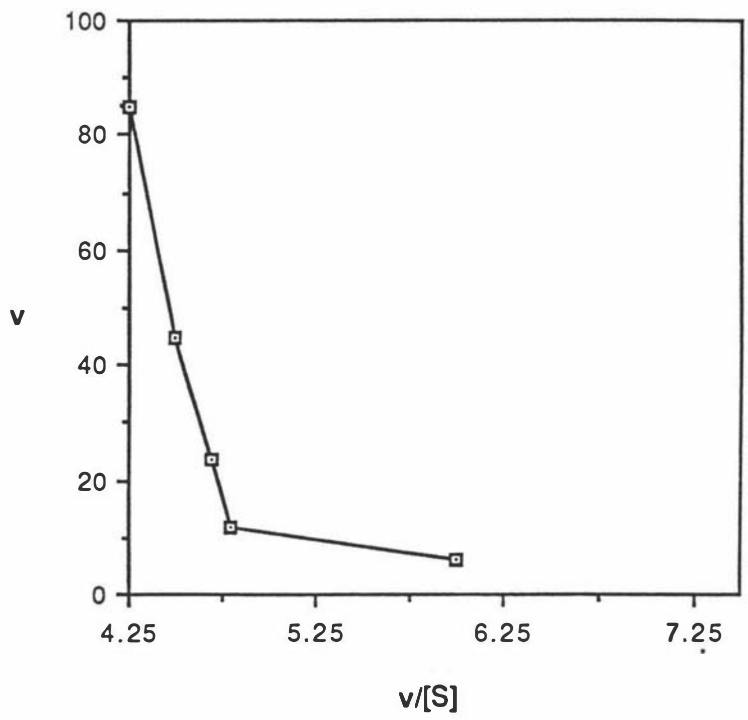
v/Vmax-v
10^1
10^0
10^-1
10^-2
10^-1
10^0
10^1
10^2
[S]



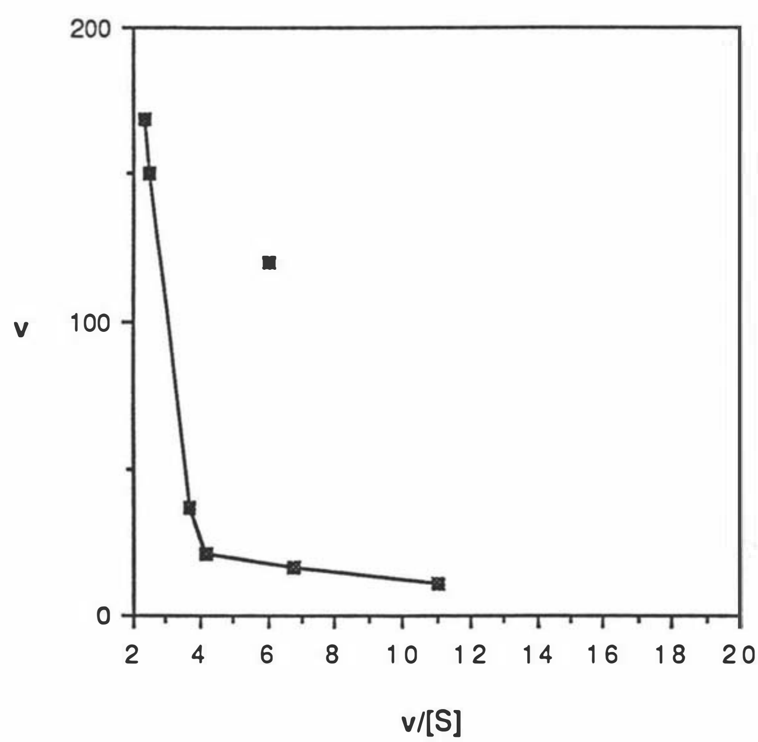
v/Vmax-v
10^0
10^-1
10^-2
10^-1
10^0
10^1
[S]

Figure 26: Eadie-Hofstee plot of glucose uptake by *S. malidevorans* 442.

Figure 27: Eadie-Hofstee plot of glucose uptake by mutant 11.



100
80
60
40
20
0
v
4.25
5.25
6.25
7.25
v/[S]



200
100
0
v
2
4
6
8
10
12
14
16
18
20
v/[S]

was 28.6 nmol/min/mg dry weight. Eadie-Hofstee plots for malate uptake by mutant 11 and wild-type but were neither obviously monophasic nor biphasic. Hill plots for the wild-type and mutant 11 have slopes of approximately one, indicating that malate binding is not cooperative (Figures 24 and 25).

Eadie-Hofstee plots for glucose uptake by both *S. malidevorans* 442 and mutant 11 indicate the presence of more than one glucose transporter (Figures 26 and 27). The apparent K_m's for the high and low affinity transporters of *S. malidevorans* 442 were 2.1 mM and 17.3 mM, respectively. The apparent K_m's for the high and low affinity transporters of mutant 11 were 1.3 mM and 44.2 mM, respectively. The V_{max} for glucose uptake by *S. malidevorans* and mutant 11 were 85 and 185 nmol/min/mg dry weight, respectively. Hill plots for glucose uptake by *S. malidevorans* 442 and mutant 11 were not linear and, therefore, are difficult to interpret.

10. Molecular genetics

The characterisation of the gene(s) involved in the mutant 11 phenotype would be aided by cloning the gene(s) for malate dependence. Since the mutation is recessive gene libraries were made of the wild-type, *S. malidevorans* 442, using suitable yeast bacterial shuttle vectors.

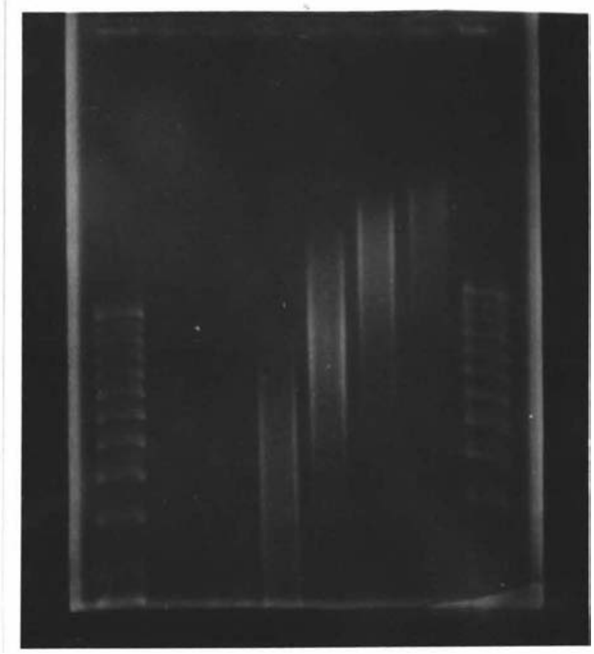
10.1. Construction of the pFL20 *S. malidevorans* gene library

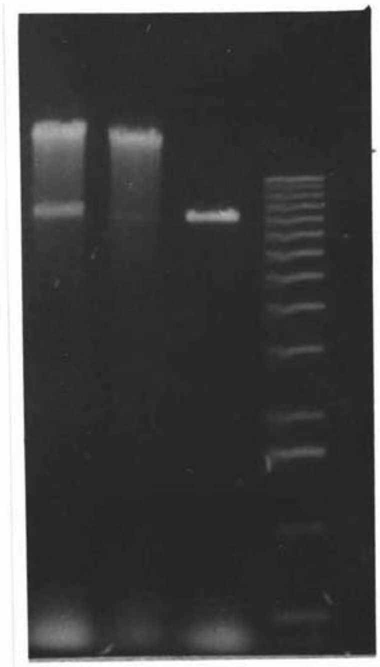
pFL20 was prepared by transforming *E. coli* HB101 with a sample of plasmid and selecting for colonies that were resistant to ampicillin. Transformation frequency by this method was routinely 1 x 10^6 cells/µg DNA.

Bulk plasmid DNA was prepared and completely digested with *Bam H1*. The plasmid was treated with alkaline phosphatase to prevent re-ligation of plasmid ends and protein was removed by phenol-chloroform extraction followed by ethanol precipitation to extract the DNA. Pure *S. malidevorans* 442 DNA was partially digested with *Sau3A* at 37°C for a range of times, pooled and then separated according to size in a NaCl gradient (Plate 8). Fractions 6,7, and 8, which contained most of the DNA fragments between 5-20 kb, were pooled and used in ligations with digested, CAP treated pFL20. Ligations were checked by gel electrophoresis for the disappearance of the linear plasmid band (Plate 9). *E. coli* HB101 competent cells (BRL) were transformed with the ligated DNA and grown on plates containing ampicillin. The transformation frequency was 5 x 10^3 transformants per µg ligated DNA, compared with the 5 x 10^7 transformants per µg of pBR322 monomer supplied. The percentage of colonies carrying plasmids containing insert DNA was determined by checking the transformants on plates containing tetracycline. Colonies with plasmids with inserts are rendered tetracycline sensitive. Approximately 50% of the transformants were tetracycline sensitive, and the average insert size was approximately 5.4 kb (Plate 10). A total of 11,000 colonies

Plate 8: NaCl fractions of endonuclease digested *S. malidevorans* 442 DNA on an agarose gel, flanked by BRL ladder.

Plate 9: Ligation of pFL20 and *S. malidevorans* 442 DNA. Lane 1, conditions not favouring plasmid insert ligation. Lane 2, optimum ligation conditions for plasmid insert ligation; Lane 3, unligated pFL20 DNA; Lane 4, BRL ladder





were collected. The completeness of the library was calculated as approximately 80%, using the largest estimate for the *S. pombe* genome size (Smith *et al.* 1987)

A uracil requiring *S. pombe/S. malidevorans* hybrid in which the malate dependence originated from mutant 11 was not obtained in this or another study (Lubbers 1987). XN1 a (*ura 4*, mig$^-$), a strain in which the malate dependence originates from a different *S. malidevorans* mutant, was used as the recipient strain for the screening of the *S. malidevorans* pFL20 gene library. No transformants of strain XN1 were recovered when either the lithium chloride or spheroplasting methods were used. In contrast, the parent, *S. pombe* WT 2, was successfully transformed by pFL20 using the lithium chloride method.

10.2. Construction and screening of the pDB262 *S. malidevorans* gene library

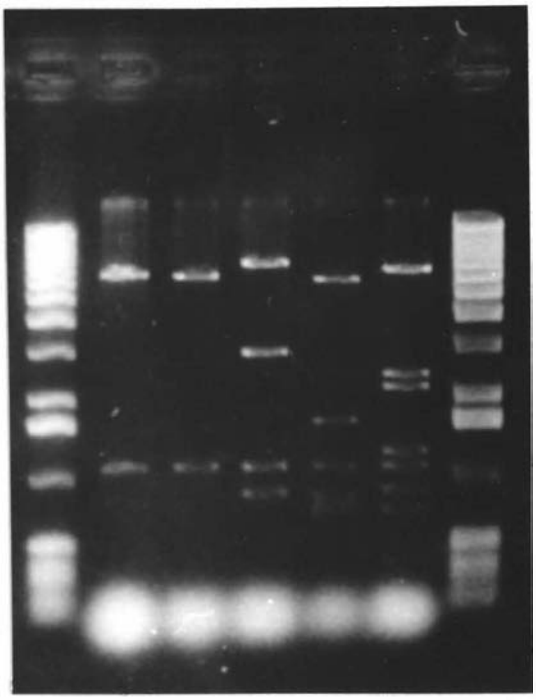
The plasmid pDB262 (Wright *et al.* 1986) was prepared by transforming *E. coli* JA221 to leucine prototrophy with a sample of plasmid. Transformation frequency by this method was routinely 1×10^6 cells/μg DNA.

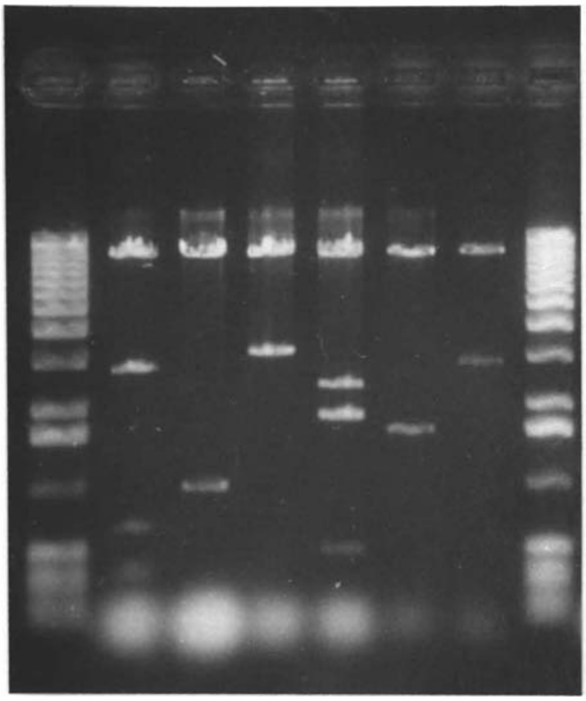
Bulk plasmid DNA was prepared and completely digested by *HindIII* (Boehringer Mannheim). Following digestion, the plasmid DNA was treated with alkaline phosphatase (Boehringer Mannheim) to prevent plasmid religation. *S. malidevorans* 442 DNA was partially digested with *HindIII* by pooling samples of DNA digested at 37^{O}C for 10, 15, 20 and 25 minutes.

Both *S. malidevorans* 442 DNA and pDB262 DNA were extracted with phenol-chloroform to remove protein and then precipitated with ethanol to extract the DNA. They were then resuspended in T.E. buffer. Ligations were done between digested plasmid and *S. malidevorans* 442 DNA. The correct ratio of vector to insert DNA was determined initially by gel electrophoresis and checking for the disappearance of the vector band after ligation, and finally, by checking the transformation frequency of *E. coli* JA221 by selecting for tetracycline resistant colonies. Only those cells that bear plasmid containing inserts are able to grow on tetracycline. Multiple ligations were done and this DNA was used to transform JA221. Transformation frequency was low at approximately 1 x 10^3 /µg DNA. All transformants were grown on plates containing tetracycline. In order to ensure that slow growing transformants were not out-competed and to ensure the best chance for obtaining a representative gene library, transformants were plated at no more than 300 colonies per plate. A total of 10,000 colonies were collected and stored in 15% glycerol at -70^{O}C. Average insert size was determined by growing up individual, randomly selected transformants, and isolating plasmids by the rapid boil method. The average insert size was found to be approximately 3.0 kb (Plate 11). The library was estimated to be approximately 80% complete using the largest estimates of the *S. pombe* genome (Smith *et al.* 1987).

Plate 10: HindIII digests of plasmids extracted from five randomly chosen colonies from the construction of the pFL20 gene library, flanked by BRL ladder. Lanes 2 and 3 show plasmid without insert.

Plate 11: HindIII digests of plasmids extracted from six randomly chosen colonies from the construction of the pDB262 gene library, flanked by BRL ladder.





XW1 15 was used as the recipient strain for the screening of the *S. malidevorans* 442 pDB262 gene library, using the lithium method. Transformation frequency was routinely 1 x 10^3 transformants per μg DNA. Approximately 40,000 transformants were screened for malate independence without success. Approximately 40,000 transformants were screened for adenine prototrophy without success.

10.3. Screening of a *S. pombe* gene library

A *S. pombe* gene library in pDB262, obtained from P. Nurse, was screened for clones that complement the mutant 11 mutation. XW1 15 (*ade 6, leu 1,* mig$^-$) was used as the recipient strain and was transformed using the lithium method. A total of 40,000 transformant colonies were screened for malate independence by being plated on media without malate. No malate independent transformants were found. A similar number of transformants of XW15 were screened for adenine prototrophy, also without success.

Discussion

1. Taxonomy of *Schizosaccharomyces pombe* and *Schizosaccharomyces malidevorans*.

S. malidevorans was first described by Rankine & Fornachon (1964). *S. malidevorans* differed from *S. pombe* in that it produced spores with a rough outer membrane and could not ferment maltose. These differences and its ability to decompose L-malic acid (malate) completely in all media tested led these workers to propose the isolate as a new species. Johannsen (1981) reported that *S. malidevorans* and *S. pombe* were not interfertile. However, other workers were able to successfully cross the two species (Sipiczki *et al.* 1982). Slooff (1970) reported that *S. pombe* frequently possessed ascospores with rough outer membranes like *S. malidevorans.* The increased decomposition of malic acid and inability to ferment maltose were not considered sufficient differences to separate the two species (Bridge and May 1984). The current consensus is that *S. malidevorans* should be regarded as a strain of *S. pombe* (Bridge and May 1984, Sipiczki *et al.* 1985, Sipiczki *et al.* 1982, Yamada *et al.* 1973, Yamada *et al.* 1987).

Mutant 11 was isolated from *S. malidevorans*, but attempts to obtain similar mutants from *S. pombe* had failed (Lubbers 1987). This cast doubt on whether *S. malidevorans* and *S. pombe* should be regarded as members of the same species.

Results reported in this thesis show that the malate dependent phenotype was only found in spore clones with the homothallic mating-type (Table 11). Since homothallism is apparently required for the maintenance of the malate dependent phenotype it is possible that a homothallic character is required for the isolation of malate dependent mutants. Isolation of malate dependent mutants from homothallic *S. pombe* strains confirmed this contention. Furthermore, the use of heterothallic strains explained the failure of the previous attempt to isolate these mutants from *S. pombe* (Lubbers 1987).

There are conflicting reports on whether *S. malidevorans* is able to grow on, i.e. assimilate, maltose as a sole carbon source. In the original description of the species, *S. malidevorans* was reported to assimilate maltose (Rankine & Fornachon 1964). However, two taxonomic texts (Barnett *et al.* 1983, Yarrow 1984) report that *S. malidevorans* is unable to assimilate maltose. More recently Bridge & May (1988) reported that the *S. malidevorans* type strain at the Australian Wine Research Institute (AWRI 158) was homothallic and able to assimilate maltose. They claimed that the type strain at the Centraalbureau voor Schimmelcultures (CBS 5557) was heterothallic, supporting work by Sipiczki *et al* (1982), and unable to assimilate maltose. Their conclusion was that maltose assimilation is under the control of the mating-type locus in *S. malidevorans*.

After further investigations, Sipiczki *et al.* (1985) claimed that *S. malidevorans* CBS 5557 was homothallic, contradicting their earlier report. Also, the two taxonomic texts, Barnett *et al.* (1983)

and Yarrow (1984), both mention CBS 5557's ability to form spores, implying that *S. malidevorans* is homothallic.

In this study *S. malidevorans* 442 (AWRI 158) was found to be homothallic. *S. malidevorans* 442 failed to assimilate maltose, confirming the taxonomic texts (Barnett *et al.* 1983, Yarrow 1984). Neither homothallic nor heterothallic lines of *S. malidevorans* 442 were able to assimilate maltose. This indicates that the ability to assimilate maltose is not under the control of mating-type in this *S. malidevorans* strain, contradicting the findings of Bridge and May (1988).

2. Investigation of the mutation(s) that result in the malate dependent phenotype

The malate dependent mutants from both *S. malidevorans* and *S. pombe* were isolated with a frequency of approximately one mutant per four thousand surviving colonies (Rodriguez and Thornton 1989, this study), when the kill was approximately 90%. The high frequency suggests that the mutant phenotype is likely to be the result a single mutagenic event. The frequency of red adenine mutants, obtained by UV mutagenesis in both species in this study, was approximately the same as for malate dependent mutants. This suggests that malate dependence, like the red adenine phenotype (Phipps *et al.* 1985), can be caused by mutation of more than one gene. This is supported by the finding of more than one complementation group of malate dependent mutants (Parker 1988; Rodriguez, personal communication; Hansen, personal communication). Another possibility is that the malate dependence locus is a 'hot spot' for mutation (Ayala and Kiger 1984).

Fermentation trials and other tests showed that class 1 revertants were altered in all the characteristics associated with mutant 11. Some characteristics were restored to the wild-type level and some were only partially restored (Tables 5, 18 and Figure 18). The frequency of class 1 reversion of mutant 11 is approximately 1 x 10^{-7}, which is similar to the frequencies of reversion of many single mutations. This observation suggests that all phenotypes are effected by a single gene mutation.

Class 2 revertants show a change in most but not all phenotypic characteristics associated with mutant 11 (Tables 5 , 18 and Figure 18). They were isolated at a frequency of 1 x 10^{-7} suggesting that they were the result of a single mutational event. Class 2 revertants were the only strains which showed many similarities to mutant 11 including blue colour on indicator plate (Table 5). Partial revertants may arise by reversion of one of several mutations involved in a mutant phenotype.

These data suggest that malate dependence is caused by a single genetic event. The result of complementation tests between *S. pombe* mutants and a mutant 11 derivative, XL1 b (Table 17), although tentative, suggest that there is more than one locus that, when mutated, could give rise to the malate dependent phenotype. There also appears to be more than one complementation group amongst the different *S. malidevorans* mutants (Rodriguez personal communication, Hansen personal communication, Parker 1988).

This suggests that malate dependence can arise by single mutations in one of a number of different genes.

The differences in the phenotype of mutant 11 and WT 6 support the possibility of more than one complementation group. Mutant 11 uses malate faster than the wild-type as well as depleting the medium of malate almost completely (Figures 1 and 2). WT 6 uses significantly more malate than the wild-type but does not, initially, appear to use malate faster than the wild-type (Figures 3 and 4). Increased malic enzyme activity in mutant 11, but not WT 6, could account for faster utilisation of malate by mutant 11 than the wild-type.

Another difference is that the inhibition of glucose uptake by malate in *S. malidevorans* 442 has been lost by mutant 11 (Figures 18 and 19) while WT 6 retains this inhibition (Figures 20 and 21).

Class 2 revertants were isolated from mutant 11, but not isolated from WT 6. Lack of these revertants is most easily explained if mutant 11 is altered in a different gene to WT 6.

The mutation appears to be recessive because diploids derived from crosses between malate dependent and malate independent strains, could be selected on minimal plates which lacked malate.

Mutant 11 was found to have a different karyotype, by TAFE, to that of *S. malidevorans* 442 (Murray 1989). The appearance of an apparently very large chromosome could be due, in part, to an alteration in the structure of the chromosome. The formation of a ring chromosome 2, containing extra DNA, in *S. pombe* resulted in a karyotype with only two bands at the positions of chromosomes 1 and 3 (Rochet and Fan 1990). These workers found that diploids heterozygous for the ring chromosome were unstable. If mutant 11 contained a ring chromosome the very low spore viability and abberant segregation patterns observed in crosses with *S. pombe* strains could be explained. However, DNA content and UV resistance studies (Murray 1989) provided evidence that the karyotype of mutant 11 reflects a large increase in DNA content, consistent with the appearance of a very large band in mutant 11. These data suggest that mutant 11 has undergone a large chromosomal rearrangement, including duplication of a large part of the genome. Similar karyotypes were found in other malate dependent mutants of *S. malidevorans* (Hansen personal communication), suggesting that the rearrangement is specific. The rearrangement co-segregates with malate dependence, as is shown by the hybrid strains derived from mutant 11 (Plate 6).

Two class 1 revertants, which were shown to have a phenotype similar, but not identical, to the wild-type, also have karyotypes similar but not identical to the wild-type (Plate 6). The size of band 2 appears to be considerably smaller than the new band in mutant 11, suggesting that class 1 revertants are due to the loss of part of the extra DNA of mutant 11. Class 2 revertants have also lost part to the extra DNA but less than the class 1 revertants. Malate independent hybrids, like revertants of mutant 11, have karyotypes in which part of the extra DNA has been lost. One of these hybrids (XW1 2) has two bands between chromosomes 1 and 3 (Plate 6). Since aneuploids are often non-viable (Niwa and

Yanagida 1985), the apparent fourth chromosome in this strain is possibly due to instability of the chromosomal rearrangement. The large proportion of nonviable spores may have had lethal combinations of chromosomes and rearranged chromosomes. Malate dependent mutants isolated from *S. pombe* strains do not have the same karyotype as mutant 11. However, both WT 6 and WT 8 appear to have chromosome rearrangements (Plate 7).

These data are consistent with the theory that the malate dependent phenotype is caused by chromosomal rearrangement and that malate independence is restored by a further rearrangement involving loss of at least part of the extra DNA gained by the mutants.

3. Genetic analysis

Genetic analysis was undertaken to obtain strains suitable for cloning and to obtain mapping data. The segregation of markers from the cross, XL1 b (*ade 6-704,* mig^{-}) and *S. pombe* 122 (*leu 1, his 2*), did not give the expected 1:1 ratios (Table 9). Tetrad analysis of diploids from the same cross showed that spore viabilities were very low. The frequency of viable spores was one per ascus in only 50% of asci. It was, therefore, not possible to analyse all the markers from one diploid. Another study reported low spore viability in crosses between *S. pombe* and *S. malidevorans* (Sipiczki *et al.* 1982). The spore viability was higher than that found in this study. Unlike this study, expected segregation patterns from random spore analysis were obtained by them.

Despite the complex segregation pattern and low spore viability, linkage analysis of the random spore data showed that genes known to be linked in *S. pombe* were apparently linked in this cross (Tables 10 and 12). However, because of distortion of ratios, no conclusions can be reached about the possible linkage of malate dependence to any other marker.

The segregation of markers in the cross, XL1 b (*ade 6-704,* mig$^-$) and *S. pombe* 122 (*leu 1, his 2*), was much closer to 1:1 than any crosses involving mutant 11 (Lubbers 1987). In order to overcome possible incompatibility between *S. pombe* and *S. malidevorans* a malate dependent hybrid, XW1 9, from the cross described above, was mated with *S. pombe* WT 2 (*ura 4*). The spore viability was as low in this cross as it was in the cross described above. Also the ratios of homothallic to heterothallic, and uracil prototrophs to auxotrophs was distorted. These observations suggest that either the incompatibility has persisted through these crosses, or the malate dependent mutation is at least partially responsible for the low spore viability.

Low spore viability could partially be explained if the combination of malate dependence and heterothallism is lethal. The consequence in the cross, XL1 b (*ade 6-704,* mig$^-$) and *S. pombe* 122 (*leu 1, his 2*), (Table 9) would be recovery of only half the expected number of malate dependent spores. The *his 1* and *leu 2* markers both originated from the heterothallic *S. pombe* parent and are both closely linked to the mating-type locus. The effect of the nonviability of heterothallic malate dependent spores would be to reduce the numbers of both markers, although they should still be present in greater numbers than malate dependent spores. The number of spores carrying the marker closest to the mating-type region would be expected to be lower than the marker further away because of the lower frequency of recombination between the two loci (Sturtevant 1913). The results (Table 9) show that the number of malate dependent spores is low. Although less than half the expected number, it is consistent with the theory. The number of spores that carry the *his 1* marker, which is closest to the mating-type locus, is less than the number of spores carrying the *leu 2* marker. The number of spores that are heterothallic is also low (Table 9). These results are consistent with the theory that one mutant locus and homothallism are both required for the malate dependent phenotype or that the two loci are linked.

Another explanation, for low spore viability and the abberant segregation patterns, is that there could be tendency for the chromosomes derived from the *S. pombe* parent to be lost. This may be due to non-genetic imprinting, for example by methylation, allowing recognition by the cell of 'foreign' DNA. The surviving spores would tend to contain markers that were derived from the malate dependent parent. The result of this would be that any auxotrophic marker introduced for the first time into malate dependent mutants would be poorly represented in the progeny. This theory is consistent with the segregation of all markers introduced from *S. pombe* into malate dependent strains. *Leu 1*, *his 2* and *ura 4* when initially introduced (Table 9 and 14) were under-represented. Under-representation of the *S. pombe* marker is also true for the *ade 6-704* marker when initially crossed with mutant 11 (Lubbers 1987).

Once the markers are present in the malate dependent strains, the chromosomes, or part of the chromosomes, would then be recognised as '*malidevorans*' DNA. These markers should be recovered in higher numbers from progeny from crosses involving malate dependent strains carrying them. The frequency of progeny auxotrophic for leucine increased in the cross XW1 9 (mig$^-$, *leu 1*) and *S. pombe* (*ura 4*) (Table 14). Adenine requiring progeny were also abundant in the cross XL1 b (*ade 6-704*, mig$^-$) and *S. pombe* 122 (*leu 1, his 2*).

4. The involvement of mating-type in malate dependence of *Schizosaccharomyces*

The homothallic character was required in order to obtain malate dependent mutants. All malate dependent *S. pombe/S. malidevorans* hybrids are homothallic, suggesting that homothallism is also required for the maintenance of malate dependence through crosses with heterothallic strains. The requirement for homothallism suggests that strains require the ability to switch mating-type in order to isolate malate dependent mutants from them.

Comparison of the mating-type system of *S. pombe* and *S. malidevorans* indicated that, although not identical, they are very similar (Sipiczki *et al.* 1985). Mating-type switching in *S. pombe* is a process that involves copy transposition of DNA from 'silent cassettes', i.e. the DNA is not transcribed, (*mat2* and *mat3*), into a site where they are transcribed (*mat1*) (Beach and Klar 1984). Switching of the mating-type involves switching (*swi*) genes. The *swi* genes are involved in making a double stranded cut in the genome close to *mat1* which signals the start of mating-type switching, initiation and resolution of copy transposition (Egel *et al.* 1984, Gutz and Schmidt 1985, Schmidt 1987).

The DNA sequence of the mating-type region and the presence of double stranded cuts causes a destabilisation of the region, both mitotically and meiotically. Errors in recombinations can lead to many different rearrangements. Many heterothallic strains have arisen through deletions or duplications of part of the mating-type region (Beach and Klar 1984).

The evidence suggests that malate dependent mutants have chromosomal rearrangements. The involvement of the mating-type region in obtaining these mutants suggests that the rearrangements may be due to UV induced errors in mating-type switching. The karyotypes of mutant 11 and derivatives of mutant 11 indicate changes in chromosome 2, which contains the mating-type region. Only cells which can switch their mating-type could be mutated by such a mechanism, explaining the requirement for homothallism in order to isolate malate dependent mutants.

Abundant malate independent homothallic spores were isolated from the crosses XLb 1 (*ade 6-704*, mig$^-$) and *S. pombe* 122 (*leu 1, his 2*), and from WT1 9 (*leu 1*, mig$^-$) and WT 2 (*ura 4*) (Tables 10 and 15). If *mat1* was the site of insertion of the extra DNA, malate dependence and homothallism would have co-segregated, unless the mating-type region had been duplicated.

A large chromosomal rearrangement involving duplication and transposition into a different site could cause the DNA close to the insertion to be regulated differently than in the wild-type, similar to position-effect variegation (Reuter and Wolff 1981). The altered control of these genes could be responsible for the malate dependent phenotype. Evidence supporting such a model is that class 1 revertants have karyotypes that suggest that they have lost most of the DNA carried by mutant 11 (Plate 6). The abnormal control of most of the duplicated genes could then be lost. Class 2 revertants may have lost only enough DNA to render them malate independent but not enough to restore other wild-type functions. Alternatively a gene or regulatory element could have been disrupted by the insertion of DNA.

Mating is initiated in *S. pombe* in response to starvation conditions (Egel 1989). Mutant 11, although homothallic, does not appear to be able to form zygotes or sporulate in pure culture. Mutant 11 and other malate dependent mutants appear to mate poorly with any other strain, although once diploids are formed abundant azygotic asci are formed. The apparently reduced ability of malate dependent strains to conjugate might suggest the involvement of genes in the mating process, such as sterility (*ste*) genes (Thuriaux *et al.* 1980, Girgsdies 1982, Micheal and Gutz 1987). However the phenotype of mutant 11 does not resemble that of any of the known mutations in mating. Conjugation in malate dependent strains is probably affected by other functions.

The results presented in this thesis indicate that malate dependent mutants are probably defective in glucose metabolism. Although there appears to be a requirement for homothallism for the isolation of these mutants, it may be that homothallism is required only to obtain them with a very high frequency associated with chromosomal rearrangements. It may be possible to obtain very rare malate dependent mutants, from heterothallic strains, that do not have chromosomal rearrangements. These mutants could then be used to obtain an improved genetic analysis of malate dependence.

5. The uptake of glucose and malate by *S. malidevorans* and *S. pombe*

The K_m of malate uptake by *S. malidevorans* 442 is very similar to the K_m reported for an *S. pombe* strain (Osothsilp and Subden 1986a), supporting the similarity between the two species. Malate uptake by *S. pombe* WT 4 and *S. malidevorans* 442 was inhibited by glucose (Figures 12, and 14). Inhibition of malate uptake by glucose in *S. pombe* has been reported previously (Osothsilp and Subden 1986a). This finding implies, firstly, that glucose is not required directly for the transport of malate and secondly, that malate uptake is subject to control by glucose. Glucose may be required to induce the malate uptake system, or perhaps simply to provide energy for the active transport of malate.

An alternative possibility is that there is a common transport system for malate and glucose, induced by glucose, would explain glucose dependent malate utilisation. It could also explain the apparent alteration in the kinetics of both malate and glucose uptake. Malate and glucose would compete for the transport system, explaining the inhibition of malate transport for glucose and vice versa (Figures 12, 14, 19 and 21). However, most of the evidence on malate and glucose transport in yeast suggests that a common transport system for both substrates is unlikely. Malate is actively transported in *S. pombe* by a common dicarboxylic acid transport system (Osothsilp and Subden 1986a) as it is in other yeast and certain bacteria (Zmijewski and MacQuillan 1975, Corte-Real *et al.* 1989, Wolfinbarger and Kay 1973, Ronson *et al.* 1981, Hornez *et al.* 1989). The inhibition of malate uptake by succinate in both *S. pombe* WT 4 and *S. malidevorans* 442 supports this finding (Figures 15 and 16). Herzberger and Radler (1988) explored but discounted the possibility of a common transport system for malate and glucose in *Z. bailii*. No other dicarboxylic acid transport system was reported to transport glucose.

The mechanism of glucose uptake in *S. pombe* has not been thoroughly studied, and there are no reports on glucose uptake by *S. malidevorans*. Hofer and Nassar (1987) reported that glucose is actively transported in *S. pombe* by an H^+-symporter. Under anaerobic conditions there is insufficient energy for active transport, but the symporter still allows facilitated diffusion of glucose. If both malate and glucose are actively transported, they may compete for H^+ ions, thereby explaining the inhibition of malate uptake by glucose and vice versa.

In this study, there is evidence for more than one glucose transporter in *S. malidevorans*. Eadie-Hofstee plots of glucose uptake by *S. malidevorans* 442 and mutant 11 indicate the presence of two transporters of different affinities (Figures 26 and 27). Two glucose transporters are found in several yeast species (Does and Bisson 1989, Barnett and Sims 1976a, Barnett and Sims 1976b). The K_m values of high affinity transport of mutant 11 and the wild-type (1.3 mM and 2.1 mM, respectively) were similar to the K_m of high affinity transport (2mM) found in *Sacch. cerevisiae* (Does and Bisson 1989).

The K_m value of low affinity transport in *S. malidevorans* 442 an in mutant 11 were probably not significantly different. The Km of low affinity transport in *S. malidevorans* 442 was very similar (17.3 mM) to that of *Sacch. cereviseae* (20mM). The kinetics of malate and glucose are apparently unaltered in mutant 11, arguing against structural alterations in transport proteins. However there appear to be some changes in the patterns of malate and glucose uptake and further work would be useful to investigate these differences. The inhibition of malate uptake by glucose may be due to competition between the different molecule for the dicarboxylic acid transporter, even if glucose is not subsequently transported. The kinetics of the malate transporter in the presence of would ascertain the type of inhibition involved. Alternatively, glucose metabolism may inhibit malate uptake, like it appears to in *Z. bailli* (Herzberger and Radler 1988). If this is the case, the glucose analogue 2-deoxyglucose should not inhibit malate uptake. Further work should be done to verify whether either glucose or malate transport, or both are structurally altered.

6. Models to explain malate dependence

Malate dependence is a previously unreported phenotype (Rodriguez and Thornton 1989). The ability to isolate malate dependent mutants with high frequency plus the evidence for more than one complementation group and the differences in phenotype of mutant 11 and WT 6 suggest that malate dependent mutants may be obtained by mutation in more than one gene.

Malate dependence is accompanied by increased utilisation of malate (Figures 2 and 4). The K_m of malate uptake is higher in mutant 11 compared to the wild-type. However, this apparent alteration does not affect the total amount of uptake by mutant 11 (Figure 11). Malate uptake by WT 6 is also unaltered compared to the wild-type (Figure 13).

Malic enzyme activity is significantly increased in mutant 11 compared to the wild-type. The heat inactivation of malic enzyme activity in mutant 11 suggests either that there are two types of malic enzyme with nearly identical affinity for malic acid and NAD, or that a less heat stable form of the same enzyme has been produced in mutant 11 (Figure 9). Malic enzyme is considered a gluconeogenic enzyme in *Sacch. cerevisiae* (Wills *et al.* 1986), *Neurospora crassa* (Zink 1967) and *Aspergillus nidulans* (McCullough and Roberts 1974), and as such may be a catabolite sensitive (CS) enzyme. Under catabolite derepressed conditions, i.e. low glucose concentration, perhaps a second, heat labile malic enzyme may be induced. WT 6 does not have increased malic enzyme activity suggesting that these mutants are not identical.

Under conditions that favour derepression of malic enzyme, malate transport may also be derepressed. Increased V_{max} is indicative of derepression of uptake (Novak *et al.* 1990). This may explain the higher V_{max} of malate transport by mutant 11 compared to the wild-type.

A characteristic that both mutant 11 and WT 6 share is a significantly lowered glucose utilisation compared to the respective wild-type strains (Figures 1-4). The finding of a decreased glucose uptake in both mutants suggests that the lowered utilisation is due to lowered ability to transport glucose (Figures 18 and 20). Lowered glucose uptake may explain the characteristic pH profiles of both mutants. *S. pombe,* grown in media containing malate and low glucose concentrations (0.5-2%), had pH profiles very like malate dependent mutants (Osothsilp and Subden 1986b). The altered K_m of the low affinity glucose transporter of mutant 11 suggests either that the mutation causing malate dependence affects glucose uptake directly, or that the mutation has affected the regulation of the low affinity glucose transporter.

Model 1: Altered low affinity glucose transport

The high concentrations of glucose in media used in this study would repress the high affinity glucose transport system if the regulation of glucose uptake is similar to *Sacch. cerevisiae* (Bisson and Fraenkel 1983, Bisson and Fraenkel 1984). A mutation in the gene, or one of the genes for the low affinity glucose transporter might then result in glucose uptake that is sufficiently low for the mutants to require another carbon and energy source. If glucose uptake was reduced, pyruvate concentrations would also be low (Lagunas 1986). Malate may fill the need by producing pyruvate, through the reaction of malic enzyme.

Malic enzyme is reponsible for most of the malate utilisation by *S. pombe*, either aerobically or anaerobically (Osothsilp and Subden 1986b). Malic enzyme catalyses the following reaction:

$$\text{L-malate} + \text{NAD(P)} \quad \Rightarrow \quad \text{pyruvate} + \text{NAD(P)H} + CO_2$$

Pyruvate is the common substrate for both respiratory growth, through the TCA cycle, and fermentation. The fermentation of pyruvate involves the enzymes, pyruvate decarboxylase, which mediates the formation of acetaldeyde, and alcohol dehydrogenase, which mediates the formation of ethanol. The respiration of pyruvate, via acetyl-CoA, is mediated by the pyruvate dehydrogenase complex (PDC). PDC has a higher affinity for pyruvate than pyruvate decarboxylase, so respiration is favoured at low glucose concentration. Fermentation operates under conditions that provide excess pyruvate or in the absence of oxygen (McDonald and Tsai 1989).

At the glucose concentrations of media used in this study, the TCA cycle should be fully repressed. However, if malate were required for use in the TCA cycle, succinate or other TCA cycle intermediates should relieve malate dependence. Succinate was found to compete with malate for uptake in both malate dependent mutants, suggesting that succinate is transported. Despite the apparent uptake of succinate, Rodriguez and Thornton (1989) found that succinate did not relieve malate dependence.

Transport systems are often coded for by more than one gene which would explain why there may be more than one complementation group of malate dependent mutants. However, if only low affinity glucose transport is affected then at low glucose concentrations, at which high affinity glucose transport operates in mutant 11, malate dependence should be relieved. Malate dependence was not relieved at 0.05% (2.8 mM) glucose, and is, therefore, not completely explained by this model.

Model 2: A mutation in glucose metabolism

It has been established that hexokinases and glucokinase are involved in regulation of high affinity uptake of glucose in *Sacch. cerevisiae* (Bisson and Fraenkel 1983). The regulation of low affinity glucose uptake has also been reported (Ramos *et al.* 1988). How this regulation is achieved is not known (Ramos *et al.* 1988, Does and Bisson 1989), although catabolite inactivation of the low affinity transport system of *Sacch. cerevisiae* has been reported (Busturia and Lagunas 1986). The high affinity transport of mutant 11 was not significantly different from the wild-type. Hexokinase activity was also unaffected in mutant 11 (Table 21), which might be expected if the high affinity glucose is unaffected. Since glycerol metabolism does not require hexokinase activity (McDonald and Tsai 1989), mutant 11's requirement for malate for growth on glycerol (Rodriguez and Thornton, personal communication) also suggests that a mutation in a hexokinase enzyme is unlikely. In fact, the malate dependence for growth on glycerol implies that no glycolytic enzyme prior to triosephosphate isomerase, the enzyme which converts dihydroxyacetone phosphate to D-glyceraldehyde 3-phosphate, is affected.

An important regulatory enzyme susequent to the trioseisomerase reaction is pyruvate kinase, which catalyses the following reaction:

$$\text{phosphoenolpyruvate} + \text{ADP} \quad \Rightarrow \quad \text{pyruvate} + \text{ATP}$$

Pyruvate kinase, like hexokinase and the glycolytic enzyme, 6-phosphofructokinase, is repressed whenever the ATP concentration inside the cell is high (Lehninger 1977). If pyruvate kinase activity was lowered by a mutation there would be an accumulation of phosphoenolpyruvate (PEP). PEP is an inhibitor of the glycolytic enzyme phosphofructokinase (Mandelstam *et al.* 1982). An accumulation of PEP may also inhibit the low affinity glucose transport system.

Malate dependence could be explained by lowered pyruvate kinase activity which results in a requirement for another carbon and energy source. Pyruvate kinase mutants of *Sacch. cerevisiae* have been isolated. They are unable to grow on media containing glucose as sole carbon source, and must be grown in medium containing another carbon source, e.g. pyruvate (Fraenkel 1982).

Malate in the medium could compensate for a low pyruvate pool in mutant 11. Pyruvate could then enter the respiratory pathway to the TCA cycle, or be converted to ethanol via acetaldehyde.

If malate is required to provide pyruvate, then pyruvate should relieve malate dependence, provided that it can enter the cell. Pyruvate does not relieve malate dependence (Rodriguez and Thornton 1989) and does not appear to enter mutant 11 (Rodriguez and Thornton, personal communication). Pyruvate does not appear to be oxidised by *S. pombe* either (Heslot *et al.* 1970).

Pyruvate kinase mutants with defects in the structural gene and in regulatory genes have been isolated in *Sacch. cerevisiae* (Fraenkel 1982). Therefore, the finding of more than one complementation group amongst malate dependent mutants is not inconsistent with this model. However, malate dependent mutants were not obtained in *Sacch. cerevisiae* in this or another study (Rodriguez and Thornton 1989). An explanation may be that *Sacch. cerevisiae* is far less able to utilise malate. *Sacch. cerevisiae* takes up malic acid by passive diffusion (Baranowski and Radler 1984), rather than by active transport, like *S. pombe*. Also, the malic enzyme of *S. pombe* has fifteen times the substrate affinity of *Sacch. cerevisiae* (Fuck and Radler 1972, Kuczynski and Radler 1982). Malate may not be able to compensate by producing a sufficiently high intracellular pool of pyruvate in *Sacch. cerevisiae.* Therefore, pyruvate kinase mutants may have different phenotypes in different yeast species.

Pyruvate kinase is an attractive candidate for the cause of malate dependence. However, it is also possible that malate dependence could result from a defect in any enzyme in the glycolytic pathway after and including triosephosphate isomerase. These enzymes should be assayed in malate dependent mutants. If alterations of these enzymes are found to be the cause of the mutation, then there is no reason why these mutants should be strictly malate dependent. Further work should be done on relieving malate dependence by, for example, other TCA cycle intermediates.

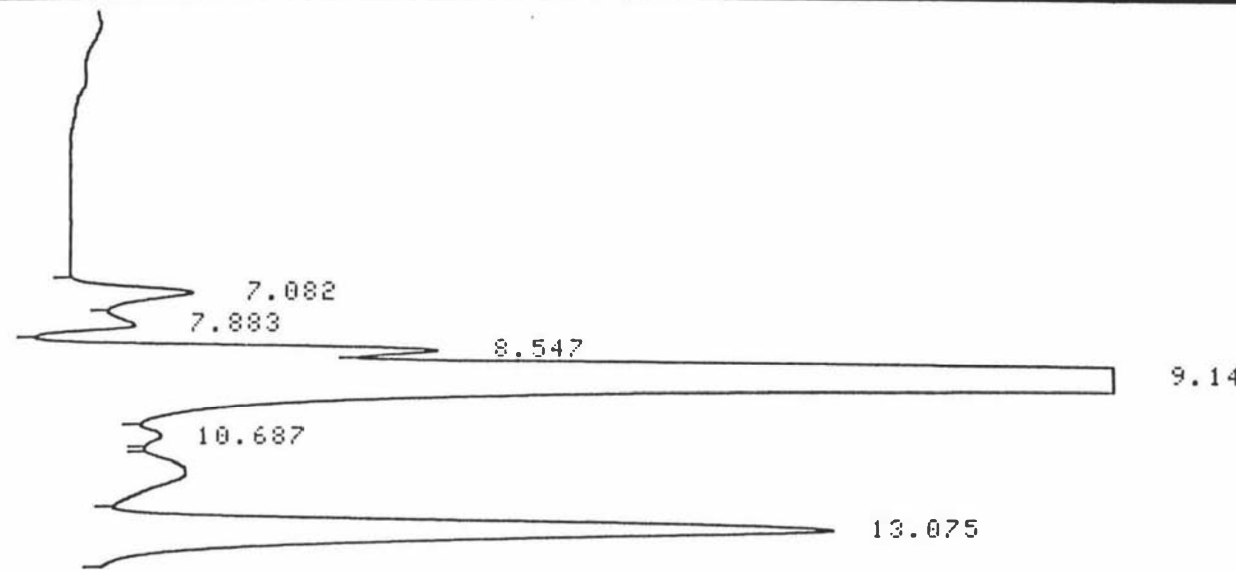
7. Conclusions

Malate dependent mutants arise from single mutational events. It appears that mutation in more than one gene can result in malate dependent mutants, with slightly differing phenotypes. The mutation is apparently recessive.

Malate dependent mutants can be obtained in *S. malidevorans* and the closely related species *S. pombe.* They can only be obtained from homothallic strains. There may a necessity for the mating-type switching event to cause a chromosomal rearrangement. Malate dependent mutants were not obtained in *Sacch. cerevisiae,* probably due to this yeast's low level of malate utilisation.

Although the phenotype involves malate dependence and increased malate utilisation, an aspect of glucose metabolism has been affected in the malate dependent mutants. Uptake of glucose is decreased in both malate dependent mutants. Whether the genes involved in glucose transport have been altered, or whether the uptake of glucose has been altered by altered regulation, is unclear. The lowered utilisation of glucose has probably derepressed malate uptake and, in mutant 11, malic enzyme activity.

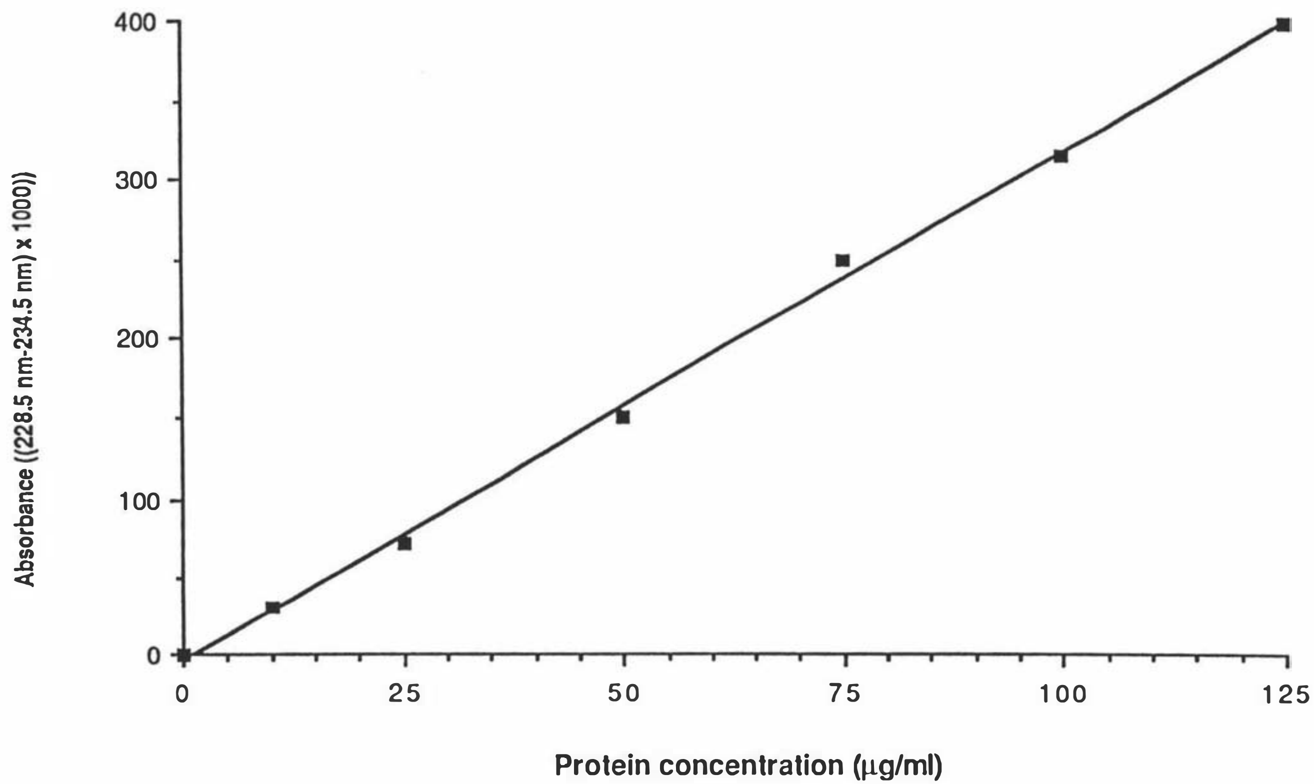
Figure A1: HPLC chromatograph; malate (mal) and glucose (glu) detected by UV detector



CHROMATOPAC C-R3A FILE 8
SAMPLE NO 0 METHOD 7
REPORT NO 9563

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	7.082	52708				
2	7.883	36034	V			
3	8.547	112978		1	11.9931	GLU
4	9.14	2202177	V	2	0.3033	MAL
5	10.687	10338	V			
6	13.075	353697				
	TOTAL	2767933			12.2963	

Figure A2: Protein standard curve (bovine serum albumin)



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