

The *mae1* Gene of *Schizosaccharomyces pombe* Encodes a Permease for Malate and other C₄ Dicarboxylic Acids

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The *mae1* gene of the yeast *Schizosaccharomyces pombe* was identified on the basis of its ability to complement a mutant defective in the transport of malic acid. Analysis of the DNA sequence revealed an open reading frame of 1314 base pairs, encoding a polypeptide of 438 amino acids with a predicted molecular weight of 49 kDa. A hydropathy profile of the predicted amino acid sequence revealed a protein with ten membrane-spanning or associated domains and hydrophilic N- and C- termini. The predicted secondary structure of the protein is similar to models proposed for other integral membrane proteins from both prokaryotes and eukaryotes. The *S. pombe mae1* gene encodes a single mRNA of 1.5 kb. The *mae1* gene is expressed constitutively and is not subject to catabolite repression as was previously reported for the malate permease systems of *Candida utilis* and *Hansenula anomala*. The *mae1* gene was mapped 2842 bp 5' to the *MFml* gene on chromosome I. Transport assays revealed that the *mae1* gene encodes a permease involved in the uptake of L-malate, succinate and malonic acid. The sequence of the *S. pombe mae1* gene is available in GenBank under Accession Number U21002.

KEY WORDS — *mae1*; malate; succinate; malonate; transport; wine; *Schizosaccharomyces pombe*

INTRODUCTION

L-Malic acid can be utilized as sole carbon and energy source by the yeasts *Candida sphaerica* (Côrte-Real *et al.*, 1989), *Hansenula anomala* (Côrte-Real and Leão, 1990) and *Candida utilis* (Cassio and Leão, 1993). The dissociated form of malate is transported across the plasma membrane by proton symports which are inducible and subject to glucose repression. However, in *Zygosaccharomyces bailii* (Rodríguez and Thornton, 1990) and *Schizosaccharomyces pombe* (Osothsilp and Subden, 1986a), L-malic acid can be metabolized in the presence of an assimilable carbon source. L-Malic acid is actively transported in the dissociated form whereas the undissociated acid enters the cell via simple diffusion (Baranowski and Radler, 1984; Osothsilp and Subden, 1986b; Sousa *et al.*, 1992). Competitive inhibition of initial uptake rates of L-malic acid by succinic acid, D-malic acid, fumaric acid, oxaloacetic acid,

α -ketoglutaric acid, maleic acid and malonic acid strongly suggests that these acids are transported by the same carrier in *S. pombe* (Sousa *et al.*, 1992).

The degradation of L-malate is of considerable technological interest to wineries. Strains of *Saccharomyces cerevisiae* can only metabolize malate to a limited extent (see Radler, 1993 for a review) and strains of *Leuconostoc oenos* are currently used to deacidify wine. Several attempts have been made to introduce the bacterial malolactic gene into *S. cerevisiae* (Ansanay *et al.*, 1993; Denayrolles *et al.*, 1995; Williams *et al.*, 1984). However, the recombinant yeast strains were unable to degrade malate effectively to L-lactate. This is not surprising as no transport system for L-malic acid has been reported for the yeast *S. cerevisiae*.

In this paper we present the first molecular characterization of a fungal dicarboxylic acid permease. Furthermore we show conclusively that the *mae1* gene product is responsible for the transport of malate, succinate and malonate in *S. pombe*.

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The *mae1* gene in *S. pombe* is expressed constitutively and is not subject to catabolite repression, as was previously shown by kinetic studies for the malate permease gene of *C. utilis* (Cassio and Leão, 1993) and *H. anomala* (Côrte-Real and Leão, 1990).

MATERIALS AND METHODS

Strains and growth conditions

Escherichia coli strain HB101 (*hsd20 leuB supE44 ara-14 galK2 lacY1 proA2 rpsL20 xyl-5 mtl-1 recA13 mcrB*) was used. Procedures for manipulating *E. coli* cells and DNA were based on Sambrook *et al.* (1989). A haploid strain of *S. pombe* 972 *leu 1-32 h⁻* (wild type), and a haploid *mae1⁻* mutant *S. pombe leu 1-32 TR⁻ h⁻ mae1⁻* (Osothsilp and Subden, 1986b) were used in this study. The yeast cells were grown in YE (2% glucose, 0.5% yeast extract), MM (Alfa *et al.*, 1993) plus leucine and YEPD medium (1% yeast extract, 2% Bactopeptone, 2% glucose), supplemented with 0.8% L-malic acid (Sigma, St Louis, MO) when required. Transformants were selected on YNB (0.17% yeast nitrogen base without amino acids and (NH₄)₂SO₄ [Difco Laboratories, Detroit, MI], 0.5% (NH₄)₂SO₄, 2% glucose, 1.7% bacto-agar [Difco Laboratories, Detroit, MI]) and malate-glucose indicator agar (MGIA), previously described by Osothsilp and Subden (1986b).

Yeast transformation

S. pombe cells were transformed by electroporation (Prentice, 1992).

Pulsed-field gel electrophoresis and Southern blotting

Chromosomal blotting was done as described by Viljoen *et al.* (1994). Standard procedures (Sambrook *et al.*, 1989) were used for Southern blotting. A 0.45 µm Hybond-N nylon membrane (Amersham International, Buckinghamshire, UK) was used. The random-primed DNA-labeling kit (Boehringer Mannheim, Mannheim, Germany) was used for radiolabeling.

Northern blotting

RNA isolation was done according to Viljoen *et al.* (1994). Total RNA was separated in a 0.8% agarose/2.2 M-formaldehyde denaturing gel and transferred to a 0.45 µm Hybond-N nylon mem-

brane (Amersham International, Buckinghamshire, UK) as described by Sambrook *et al.* (1989).

Cloning of mae1 gene

A HindIII genomic library of *S. pombe* prepared in shuttle vector WH5 by Paul Young (Queen's University, Kingston, Ontario) was used to transform *S. pombe* strain *leu1-32 mae1⁻ h⁻* according to the method of Beach *et al.* (1982). Transformants were transferred into 100 µl of MGI liquid indicator medium (Osothsilp and Subden, 1986b). Complementation was first determined colorimetrically, then confirmed by transport activity assays (Osothsilp and Subden, 1986b).

A 5.4 kb *EcoRI* subclone and a 3.4 kb *SmaI* subclone in pRS315 (Sikorski and Hieter, 1989) were transformed into the *mae1⁻* mutant to determine which fragment contained the *mae1* gene.

DNA sequence analysis of mae1

In order to sequence the cloned fragment, unidirectional digestions with Exonuclease III were performed (Sambrook *et al.*, 1989). The deletion derivatives were transformed into *E. coli* (Tschumper and Carbon, 1980).

Plasmid DNA was isolated from the transformants using the alkaline lysis method of Lee and Rasheed (1990) and digested with *PvuII* to determine the sizes of the fragments obtained. Overlapping fragments were selected for DNA sequence analysis (Tabor and Richardson, 1987) and the DNA fragment containing the *mae1* gene was sequenced in both directions using Sequenase v2.0 (US Biochemical Corp., Cleveland, OH). The nucleotide sequence was analysed with the Genetics Computer Group package of programs (Devereux *et al.*, 1984). Searches of the GenBank database were performed using the FASTA and TFASTA programs and using BLAST on the NCBI file service (Altschul *et al.*, 1990). Transmembrane segments of the *mae1* protein were predicted by the methods of Eisenberg *et al.* (1984) and Rao and Argos (1986).

Transport assays for dicarboxylic acids

Yeast cells in the logarithmic growth phase (OD of 1.2 at A₅₉₅) were harvested and washed three times with 0.1 M-KCl (pH 3.5). The cells were resuspended in 4 ml 0.1 M-KCl (pH 3.5) and stored at 4°C. Transport assays were completed within 3 h. The cell suspensions were pre-incubated for 5 min in a shaker water bath at 30°C

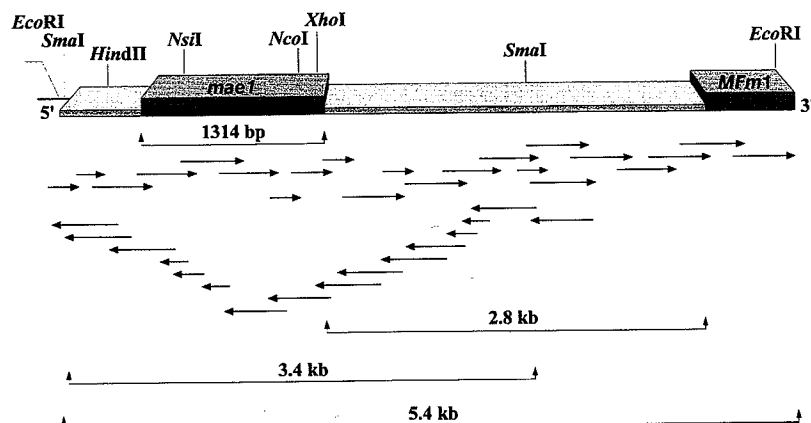


Figure 1. Restriction map and DNA sequencing strategy for the coding and flanking regions of the *mae1* gene and the *MFm1* gene. Only unique restriction sites that occur within the *mae1* gene are shown. Overlapping exonuclease fragments were generated for sequencing as indicated by the arrows. Both strands of the *mae1* gene were sequenced entirely, whereas only one strand of the *MFm1* gene was sequenced.

at 100 rpm. Assays were initiated by adding 25 μ l of 14 C-labeled L-malate (45 μ Ci/ μ mol; Amersham), 100 μ l of succinic acid (42 μ Ci/ μ mol; ICN), 100 μ l malonic acid (56.7 μ Ci/ μ mol; Du Pont) or 100 μ l α -ketoglutarate (51.8 μ Ci/ μ mol; Du Pont). A 0.5 ml sample was withdrawn at 10-, 20-, 40-, 60- and 120 s intervals, rapidly filtered through 0.45 μ m membranes (Millipore Corporation, Bedford, MA), and immediately washed three times with 5 ml amounts of ice-cold 0.1 M-KCl (pH 3.5). Filters containing the cells were oven-dried at 50°C and placed in scintillation vials containing 5 ml of scintillation reaction mixture (Boehringer Mannheim, Mannheim, Germany). Pre-boiled (5 min) cells were used to determine non-specific binding of [14 C]malate, succinate, malonate and α -ketoglutarate to the yeast cells.

RESULTS AND DISCUSSION

Cloning and subcloning of the mae1 gene

Osothsilp and Subden (1986b) generated various mutants of *S. pombe* that were unable to utilize malate. The *mae1* gene was cloned from a *S. pombe* *HindIII* genomic library by complementation of a transport mutant. A 3.4 kb *SmaI* subclone was the smallest fragment able to restore fully L-malate transport in the mutant.

Nucleotide sequence of the mae1 gene

The sequence of the *S. pombe mae1* gene is available in GenBank under accession number

U21002. A restriction map of the *mae1* gene is shown in Figure 1. The nucleotide sequence of the *mae1* gene is given in Figure 2. DNA sequence analysis revealed an open reading frame of 1314 bp. Homology searches of the GenBank database v72.0 conducted for the nucleotide sequence and the deduced protein sequence, did not reveal any significant similarity to other DNA sequences or proteins. In particular, no significant sequence homology was detected when the *mae1* protein was compared to a recently published renal sodium/dicarboxylate cotransporter (Pajor, 1995). A prominent TATAT sequence (repeated four times) was located at -153 to -175 bp of the ATG codon. A direct 10 bp repeat, TCATTTTTA, separated by 9 bp was found at positions -258 to -267 and -277 to -286.

Features of the mae1 protein

The *mae1* gene is predicted to encode a protein of 438 amino acid (aa) residues with a predicted molecular weight of approximately 49 kDa. The hydrophathy profile of the deduced aa sequence (Figure 3) revealed a protein with hydrophilic N- and C-termini and ten putative membrane-spanning helices, typical of membrane-transport proteins. No signal peptide was found at the N-terminus but the presence of an internal signal peptide should not be ruled out. Several membrane proteins, e.g. the arginine permease encoded by *CAN1* (Hoffmann, 1985) from *S. cerevisiae*, do not contain an N-terminal signal sequence.

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-378          TGATCACTATTGTTGTTCTATTTTGTCTTTCTTACTGTTGCTACACAAAATAGCTATTGTTGCTGCACCT
-300  AGACTTTTGTGGATTTCATCCTCCTACTCTGATCGGCAGTTTGCCTATTACTAAGACTAGCAACAGCGACTCATTATTTTACACTCTCATCA
-200  TTTTATTTTATTCACGAGTAACATGATGCGGATAGACTCAGAGATAAATGCTAGCAATGGTGTCTCTTCCCTCCGCTTTTCCCTTTTGG
-100  TTCCCTTTTCTCTTATATATATATATATATATATATATATATATATATATATATATATATATATATATATATATATATATATATATATAT
1   ATGGGTGAACCTCAAGAAATCTGAAACAGAGGTATCATGAGTCTGACTGGAATGCAAGCCCTCATGCCCCTCAGTCAACGACTGAGCAAT
   M G E L K E I L K Q R Y H E L L D W N V K A P H V P L S Q R L K H F 34
101  TTACATGCTGCTGGTGGTCTGACTACTGCACTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGT
   T W [ S W F A C T M A T G G V G L I I G S F P F ] R F Y G L N T [ I G K 67
201  AATTGTTATATCTTCAAACTTTTGTGTTCTCTTGGATCATGACGCTTTTGGCTTTATAAATATCCTCAACTATGCAAGGATCTCGAAC
   I V Y I L Q I F L F S L F G S C M L ] F R F I K Y P S T I K D S W N 100
301  CATCATTTGGAAAAGCTTTTCATGCTACTGTCTCTTCAATATCCAGTTCATCGACATGCTTGCATATACCCCTATCCTGATACCGGAGGTGGA
   H H L E K [ L F I A T C L L S I S Y F I D M L A I Y A ] Y P D T G E W M 134
401  TGGTGGGCTCATTGSAATCCTTTATTACATTTACGTTGGAGTATCCTTTATATACGCGTAATGGCTTTTATACATTTCAACAACATGATATAC
   V W V I R [ I L Y Y I Y V A V S F I Y C V M A F F T ] F N N H V Y T 167
501  CATTGAAACCGCATCTCCGCTGGATCTCTCTATTTTCCCTCTATGATTTGGGTGCTCATTGCGCCGCTCAATCTACAGACCCCTCATCAA
   I E T A [ S P A W I L P I F P P M I C G V I A G A V ] S T Q P A H Q 200
601  TFAAAAATATGGTATCTTGGTATCCTCTTCAAGGACTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGT
   L K N M [ V I F G I L F Q G L G F V V Y L L L F A V ] N V L R F F T Y G 234
701  GCGTGGCAACCCCAAGATCGACTGGTATGTTGTTGTCGATCCACGCTTCTCAGGTTGGGCTTAATTAATATGCGCGTGGTGGTGGTGGTGGT
   L A K P Q D R [ P G M F H F V G P P A F S G L A L I N I ] A R G A N G 267
801  CAGTGGCCCTATATTTTGTGGGCGCACTCATCGAGTATCTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGT
   S R P Y I F Y V G A [ S S E Y L G F V S T ] F M A I F I W G L A A W C 300
901  TACGTGCTGCCCATGGTGGCTTTTACGCGGCTTTTCACTCGAGCCCTCTCAAGTTTCTTGGATGGTTGCATTCATTTCCCAACGTTGGGTT
   Y C L A M V S F ] L A G F F T R A P L K F [ A C G M F A F I F P N V G F 334
1001 TTGTTAATTTGACCATGAGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT
   V N C T I E I [ G ] K M I D S K A F Q H [ F G H I G V I L C I Q W I L 367
1101 CCTAATGATTAATGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGT
   L M Y L W V ] R A F L V H D L C Y P G K D E D A H P P P K P N T G V 400
1201 CTTAAGCCCTACCTCCCACTGAAAAGCAGCTGATCTTGGAAAAGTGCATACATGTCACATCTACTGCTGGTGAATCGGATCCTCTAGTAGT
   L N P T F P P E K A P A S L E K V D T H A V T S [ T ] G G E S D P P S [ S ] 434
1301 AACATGAAGCGTTTAAAGCTGTATGCTTTTCCCTAAATTTCTCTAATAACTGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGT
   H E S V * 438
1401 CTGTATCTTATGACTACTGGTACTAATTTGCTTAGACATTTTGTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT
1501 AATAATGGTAATTTTGGTGTCTAGTGTAAATATGATGCTGCTTGCATATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT
1601 AAAATAGTATTATTTATTTATTTATCTGATTAATGCTTAAATTTATATCTTGGTCAACGACTATATCTCTTCTACTCTTATCAGCAGCAC
1701 ACTTTAGTATGTTATTTGAAAACCTGTGTATATAATCTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGT
1801 CTCBAACATATCTTGGCTATTGATGACATCTTACACACTCACTATAGTAACTAATAAATTAAGGGGATTCATGCAAAAGAACTACTAGAGTAA
1901 ACCACTATGACTAAAATAAAACTGGTAAAAGTAAATCTCAAAATATAAATCATGATAGAAAATAGTCCATTAATCAAGATAGCGTGGACGCTGACC
2001 TGATACATAGTTCACAAAGAAATAAACATCTTGAAGTAAAGCAATAGACAAATAAAGAGAGATACCTCATTTAAC

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Figure 2. Nucleotide and deduced amino acid sequence of the *mael* gene. Nucleotides are numbered on the left, and amino acids, designated by the standard single-letter codes, are numbered on the right. The arrows connecting residues 421 and 434 enclose a PEST sequence; the circled serine and threonine are the potential phosphorylation sites in the PEST sequence. The putative membrane-spanning segments are shown as solid boxes. The circled asparagines (N) are possible glycosylation sites. The residues forming a putative leucine zipper are underlined in bold. A putative 'TATA' box is underlined in the promoter region.

A structural model for the malate permease was constructed by computer analysis (data not shown). Two prominent hydrophilic linkers, 20 and 25 aa long, are located between hydrophobic

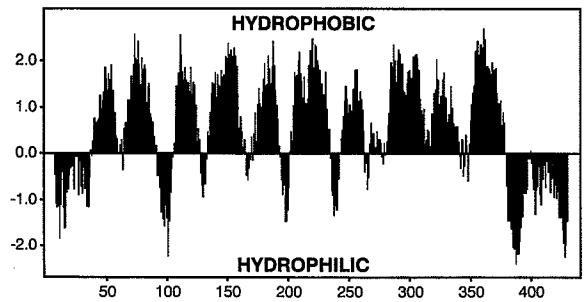


Figure 3. Hydropathy plot of the predicted *mael* protein. The profile was determined by the algorithm of Kyte and Doolittle (1982) using a window of 10 amino acids.

membrane-spanning domains two and three, and seven and eight, respectively. The lengths of the other hydrophilic linkers range from 7 to 12 aa.

Several conserved motifs were recognized in the *mael* protein. A well-conserved PEST region (aa 421–434) is found at the C-terminal end. Many proteins with intracellular half-lives of less than 2 h contain one or more PEST regions, consisting of proline (P), glutamic acid (E), serine (S), threonine (T) and to a lesser extent aspartic acid (Rogers *et al.*, 1986).

A leucine zipper motif (aa 214 to 235), consisting of four leucine residues spaced by 6 aa, is located between membrane-spanning domains six and seven. The periodicity of a leucine or isoleucine every seventh residue (Landschulz *et al.*, 1988) has been observed in several transport proteins (Bisson *et al.*, 1993). In mammalian glucose transporters and many of the fungal transporters, a conserved

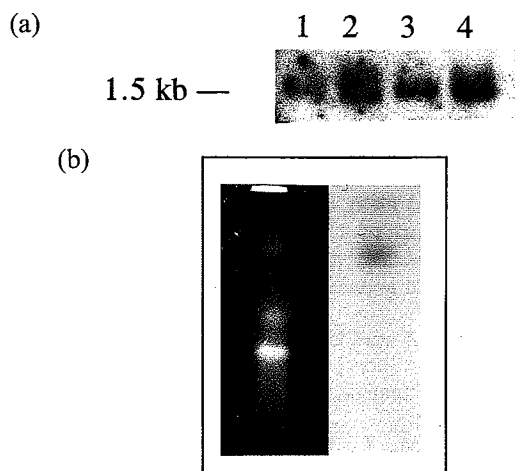


Figure 4. (a) Northern blot of wild-type *S. pombe* total RNA, probed with the 695 bp *NsiI/XhoI* fragment of *mae1*. 25 μ g of RNA was loaded onto each lane. Cells were grown in YNB medium containing 2% of either glucose (1), fructose (2), fructose buffered with 10 mM-succinate at pH 6.0 (3) or raffinose (4) as sole carbon source. (b) Chromosomal blot of the *mae1* gene. *S. pombe* chromosomes were separated on a CHEF gel (left) and probed with the labeled internal *NsiI/XhoI* fragment of *mae1* (right).

zipper motif is found in or near the second putative transmembrane domain (White and Weber, 1989). These motifs have been shown to mediate protein-protein interactions in several systems by means of a coiled-coil structure. It is not known if this motif has any function in transporters.

The *mae1* protein contains three potential N-linked glycosylation sites located at aa 193, 277 and 336. In addition, potential protein kinase C phosphorylation sites were found at positions 28 and 94.

Expression of the *mae1* gene

Northern analysis revealed that the *mae1* gene encodes a single transcript of approximately 1.5 kb. Expression of the *mae1* gene in the presence of glucose, raffinose or fructose (Figure 4a) revealed that the *S. pombe mae1* gene was not subject to catabolite repression, as was previously reported for the malate permease genes of *C. utilis* (Cassio and Leão, 1993) and *H. anomala* (Côrte-Real and Leão, 1990).

Chromosomal localization of the *mae1* gene

Southern analysis of CHEF gels (Figure 4b) confirmed the location of the *mae1* gene on chromosome I (Osothsilp, 1987). Sequence analysis

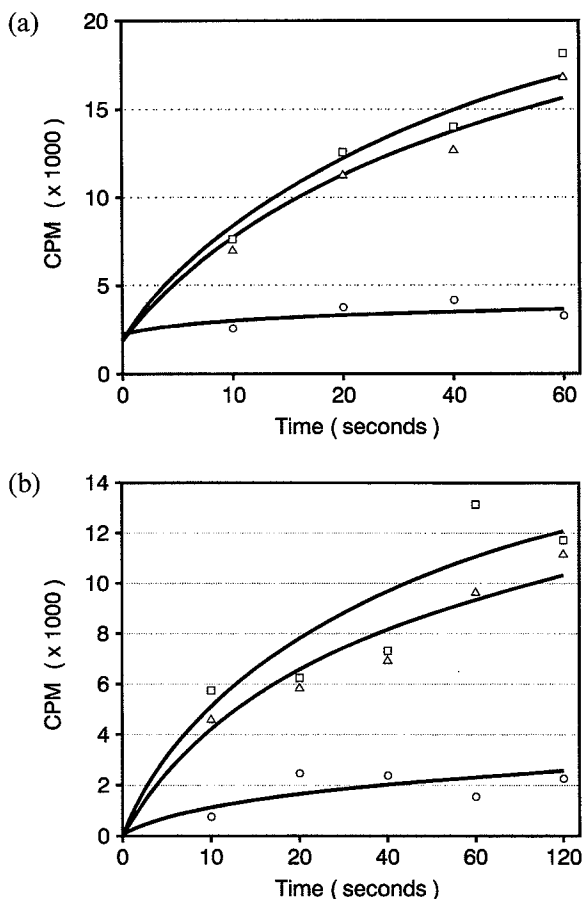


Figure 5. Uptake of [14 C]L-malic acid (a) and [14 C]succinic acid (b) by the wild-type (Δ), *mae1*⁻ mutant (\circ) and complemented mutant (\square). The transport of L-malic and succinic acid by the mutant was fully restored by transforming the cells with the *mae1* gene. Similar results were obtained when [14 C]malonic acid was used (data not shown).

revealed that the *mae1* gene is located 2842 bp 5' to the *MFml* gene (Davey, 1992; Figure 1).

Transport of dicarboxylic acids by the *S. pombe mae1* permease

Malic, succinic, malonic and α -ketoglutaric acid transport assays were done using a wild-type strain of *S. pombe*, a *mae1*⁻ mutant and the *mae1*⁻ mutant complemented with the *mae1* gene. The 3.4 kb *SmaI* fragment containing the *mae1* gene cloned into pRS315 fully restored transport of L-malic (Figure 5a), succinic (Figure 5b) and malonic acids in the *mae1*⁻ mutant. α -Ketoglutarate was not transported by any of the *S. pombe* strains used in the transport assays.

Sousa *et al.* (1992) stated that competitive inhibition of initial uptake rates of L-malic acid by succinic acid, D-malic acid, fumaric acid, oxaloacetic acid, α -ketoglutaric acid, maleic acid and malonic acid strongly suggests that these acids are transported by the same carrier. Our results conclusively show that the *mae1* gene of *S. pombe* encodes a general permease for L-malate, succinate and malonate. However, contrary to the findings of Sousa *et al.* (1992), α -ketoglutarate does not seem to be transported by the same carrier.

These data represent the first molecular genetic report on a permease for dicarboxylic acids in fungi. The co-expression of the *mae1* gene and the malic enzyme (*mae2*) gene from *S. pombe* or the malolactic gene from lactic acid bacteria in *S. cerevisiae*, could lead to the construction of wine yeast strains that should be able to degrade fully L-malate during wine fermentations.

PATENT

A provisional patent on the sequence of the *mae1* gene and its application in oenology and other commercially important processes has been filed.

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