YEAST VOL. **10:** 613–624 (1994)

Molecular Analysis of the Malic Enzyme Gene (*mae2*) of *Schizosaccharomyces pombe*

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Received 6 December 1993; accepted 6 January 1994

Sequence analysis of a 4·6-kb *Hin*dIII fragment containing the malic enzyme gene (*mae2*) of *Schizosaccharomyces pombe*, revealed the presence of an open reading frame of 1695 nucleotides, coding for a 565 amino acid polypeptide. The *mae2* gene is expressed constitutively and encodes a single mRNA transcript of 2·0 kb. The *mae2* gene was mapped on chromosome III by chromoblotting. The coding region and inferred amino acid sequence showed significant homology with 12 malic enzyme genes and proteins from widely different origins. Eight highly homologous regions were found in these malic enzymes, suggesting that they contain functionally conserved amino acid sequences that are indispensable for activity of malic enzymes. Two of these regions have previously been reported to be NAD- and NADP-binding sites.

KEY WORDS — *mae2*; malic acid; wine; *Schizosaccharomyces pombe*.

INTRODUCTION

The malic enzyme catalyses the oxidative decarboxylation of malate to pyruvate and carbon dioxide in the presence of $NAD(P)^+$ and divalent cations. The malic enzyme reaction is followed by successive decarboxylation and reduction of acetaldehyde to yield ethanol (Maconi *et al.*, 1984). It allows for the continual turnover of the tricarboxylic acid (TCA) cycle when pyruvate is limiting (Hansen and Juni, 1975) and provides a means for the oxidation of TCA cycle intermediates. Three groups of malic enzymes, depending on coenzyme specificity and ability to catalyse the decarboxylation of oxalacetate, have been reported (Figure 1). L-Malate : NAD^+ oxidoreductase (oxalacetate-decarboxylating; EC 1.1.1.38) uses NAD^+ as coenzyme, and has been found in lactobacilli (Korkes *et al.*, 1950), streptococci (London and Meyer, 1969), *Escherichia coli* (Murai *et al.*, 1972; Yamaguchi *et al.*, 1973) and *Schizosaccharomyces pombe* (Temperli *et al.*, 1965). L-Malate : NAD^+ oxidoreductase (decarboxylating; EC 1.1.1.39) is the malic enzyme most commonly found in the mitochondria of plants and animals (Artus and Edward, 1985). It has also been isolated from *Ascaris lumbriciodes* (Saz and Hubbard, 1957), *E. coli* (Murai *et al.*, 1972) and *Pseudomonas diminuta* (Suye *et al.*, 1992). This enzyme can use $NADP^+$ as a coenzyme in some cases, but prefers NAD^+. L-Malate : $NADP^+$-oxidoreductase (oxalacetate-decarboxylating; EC 1.1.1.40) is $NADP^+$-dependent and has been found in plants (Rothermel and Nelson, 1989), animals (Bagchi *et al.*, 1987), *Aspergillus nidulans* (McCullough and Roberts, 1974) and *Neurospora crassa* (Zink, 1967). Although most $NADP^+$-dependent malic enzymes can also use NAD^+, $NADP^+$ is preferred and acts as a non-competitive inhibitor of NAD^+ activity in these enzymes (Hatch and Mau, 1977).

The *S. pombe* malic enzyme has a pH optimum of 3·5–4·0 (Osothsilp and Subden, 1986a; Young, 1973) and cultures are able to metabolize up to 20 g/l malic acid (De Queiroz and Pareilleux, 1990; Taillandier *et al.*, 1988), probably due to a very high substrate affinity (Temperli *et al.*, 1965). The low K_m of 3·2 mM for the *S. pombe* malic enzyme is in contrast with the K_m value of 50 mM for *Saccharomyces cerevisiae* (Fuck *et al.*, 1973), and 15 mM for *Saccharomyces bailii* (Kuczynski and Radler, 1982).

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CCC 0749–503X/94/050613–12

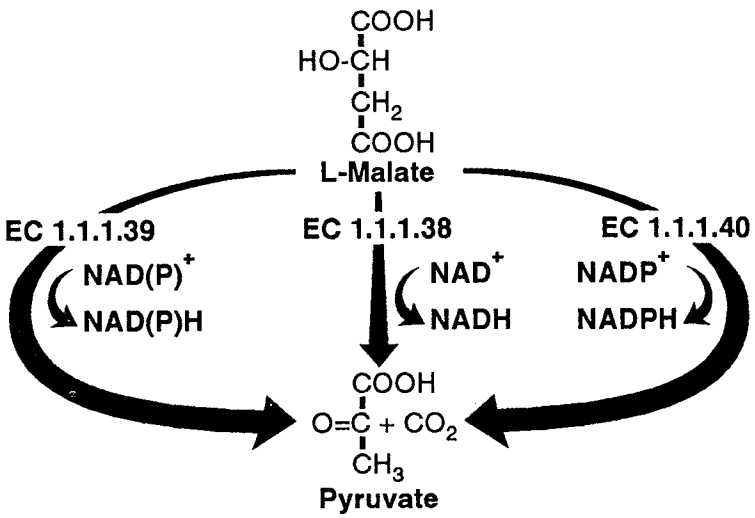

Figure 1. Malate is oxidatively decarboxylated to pyruvate via three different malic enzymes, EC 1.1.1.38-40, classified according to their coenzyme specificity. EC 1.1.1.38 and EC 1.1.1.40 are also capable of decarboxylating oxalacetate.

Taillandier and Strehaiano (1991) showed that malic acid is not integrated into biomass after its catabolism by *S. pombe*. It is completely metabolized to ethanol and CO_2 during anaerobiosis, and to CO_2 under aerobiosis (Mayer and Temperli, 1963). Malic acid degradation is glucose-dependent (Magyar and Panyik, 1989) under both aerobic and anaerobic conditions (De Queiros and Pareilleux, 1990), suggesting that the metabolism of malate requires energy, supposedly for transport of malic acid into the cell (Taillandier and Strehaiano, 1991).

Mutants of *S. pombe* strain 972h$^-$ defective in malic acid utilization (*mau*$^-$ mutants) were isolated by Osothsilp and Subden (1986a). The *S. pombe* malic enzyme gene was mapped 29 map units from the *ade6* gene on the right arm of chromosome III near the centromere (Osothsilp, 1987).

Osothsilp and Subden (1986a) reported the involvement of two additional enzymes, malate dehydrogenase and malate permease, in the metabolism of malate. Malate dehydrogenase (EC 1.1.1.37) mediates the conversion of L-malate into oxalacetate. Under anaerobic conditions, the malic enzyme alone is capable of supporting malate metabolism, although not as efficiently as in the presence of malate dehydrogenase (Osothsilp, 1987). During aerobiosis both the malic enzyme and malate dehydrogenase are required for malate utilization. The malate permease gene is constitutively expressed (Osothsilp and Subden, 1986b) and is inhibited by inhibitors of oxidative phosphorylation. The permease plays an important role in the active transport of malate.

The purpose of this study was to determine the DNA sequence of the *mae2* gene, analyse the molecular structure of the gene, and determine the size of the mRNA transcript. Furthermore, the DNA and amino acid sequences of the *S. pombe* malic enzyme were compared with those of other known malic enzymes. Several conserved regions were found in the different malic enzymes, thereby implicating possible sites involved in the malic enzyme activity.

MATERIALS AND METHODS

Strains and media

Schizosaccharomyces pombe 972 *leu1-32 h*$^-$ (wild type) and *S. pombe* 972 *leu1-32 me*$^-$ *h*$^-$ LH-67 (*mae2*$^-$ mutant) were used in this study. *S. pombe* cells were grown in YEPD medium (1% yeast extract, 2% bactopeptone, 2% glucose), supplemented with 0·8% L-malate (Sigma, St Louis, MO) when required. Transformants were selected on YNB (0·17% yeast nitrogen base without amino acids [Difco Laboratories, Detroit, MI], 0·5% $(NH_4)_2SO_4$, 2% glucose, 1·7% bacto-agar

[Difco Laboratories, Detroit, MI]) and malate-glucose indicator agar (MGIA), described previously by Osothsilp and Subden (1986a).

E. coli HB101 (*hsdS20 leuB supE44 ara-14 galK2 lacY1 proA2 rpsL20 xyl-5 mtl-1 recA13 mcrB*) was grown in TY medium (1% yeast extract, 1% NaCl, 2% tryptone) with ampicillin for plasmid preparations and in Luria Broth (0·5% yeast extract, 1% NaCl, 1% tryptone) for transformation purposes.

Yeast transformation

The *S. pombe mae2* mutant strain was cultured in YEPD and competent cells were prepared by means of the lithium acetate procedure (Moreno *et al.*, 1991). Transformants were selected on YNB plates (4 days at 30°C) and then plated on MGIA to verify their ability to degrade malic acid.

Malic acid test

S. pombe wild type, *mae2*$^-$, and *mae2*$^-$ transformed with pMV651 and pMV652, were inoculated in 10 ml YEPD containing 0·8% L-malate (final concentration 5×10^5 cells/ml), and incubated on a gyrotory shaker at 30°C for 4 days. The cells were harvested by centrifugation and the supernatant was filter-sterilized with Millex-GS 0·22 μm filter units (Millipore, Bedford, MA). The L-malic acid content of the supernatant was determined with the L-malic acid test kit (Boehringer Mannheim GmbH, Mannheim, Germany) according to the manufacturers' instructions.

Pulse-field gel electrophoresis and Southern blotting

S. pombe 972 (wild type) was grown in 100 ml YEPD until the late logarithmic phase. Chromosomal DNA plugs were prepared and the chromosomes separated by contour-clamped homogeneous electric field (CHEF) electrophoresis as described by van der Westhuizen and Pretorius (1992), with some modifications. The DNA was loaded in a 0·75% agarose (Pulsed Field Certified, Bio-Rad Laboratories, Hercules, CA) gel, and run in 0·5 × TBE buffer for 150 h at 50 V and 14°C, with a switching time of 60 min.

Standard procedures (Sambrook *et al.*, 1989) were used to prepare the gel for Southern blotting and to transfer the DNA to a 0·45 μm Hybond-N nylon membrane (Amersham International plc, Buckinghamshire, U.K.). The internal 610-bp *Eco*RV/*Ban*II DNA fragment of *mae2* was labelled with [α-^{32}P]dATP by using the random-primed DNA-labeling kit (Boehringer Mannheim GmbH, Mannheim, Germany) and used as a probe to detect the *mae2* gene.

Restriction enzyme mapping and sequencing strategy

A 4·6-kb *Hin*dIII fragment containing the *mae2* gene was cloned in the *Hin*dIII site (Subden, manuscript in preparation) of pRS315 (Sikorski and Hieter, 1989), resulting in plasmids pMV652 and pMV651 (*mae2* in reverse orientation). Plasmid pMV652 was digested with restriction endonucleases according to the manufacturers' instructions, and a restriction map was compiled.

In order to sequence the cloned fragment, unidirectional digestions with Exonuclease III were performed (Henikoff, 1984) as described by Sambrook *et al.* (1989). The deletion derivatives were transformed into *E. coli* with the Tschumper and Carbon (1980) modification of the Mandel and Higa (1970) procedure.

Plasmid DNA was isolated from the transformants using the alkaline lysis method of Lee and Rasheed (1990), and digested with *Cla*I to determine the sizes of the fragments obtained. Overlapping fragments (Figure 4) were selected for DNA sequence analysis (Tabor and Richardson, 1987). Both strands were sequenced entirely, using Sequenase v2·0 (US Biochemical Corp., Cleveland, OH), according to the manufacturers' recommendations. The GCG computer program (Devereux *et al.*, 1984) was used for the analysis of the sequence data.

RNA isolation and northern analysis

S. pombe mae2$^-$ transformed with pMV652, was cultured in 10 ml YEPD at 30°C for 48 h. Total RNA was isolated as described by Laing and Pretorius (1992), with the exception that the cells were vortexed for only 30 s. 30 μg of total RNA were separated in a 0·8% agarose/2·2 M-formaldehyde denaturing gel and transferred to a 0·45 μm Hybond-N nylon membrane (Amersham International plc, Buckinghamshire, U.K.) as described by Sambrook *et al.* (1989). The internal 610-bp *Eco*RV/*Ban*II [α-^{32}P] DNA fragment of *mae2* was used as a probe for the detection of the *mae2* mRNA transcript.

Nucleotide sequence accession number

The sequence of the *S. pombe mae2* gene is available in Genbank under accession number U00621.

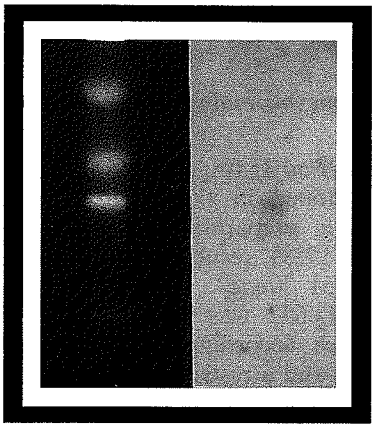

Figure 2. Chromosomal blotting of the *mae2* gene. *S. pombe* chromosomes were separated on a CHEF gel (left) and probed with the internal *Eco*RV/*Ban*II fragment of *mae2* (right).

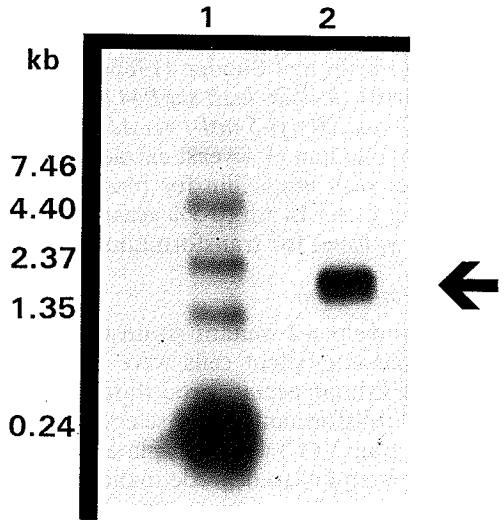

Figure 3. Northern blot of wild-type *S. pombe* total RNA, probed with the internal 610-bp *Eco*RV/*Ban*II fragment of *mae2*.

RESULTS AND DISCUSSION

Utilization of malic acid by yeast transformants

S. pombe mae2$^-$ transformed with pMV651 and pMV652 gave rise to blue-green colonies on MGIA plates after an incubation period of 4 days at 30°C. Osothsilp and Subden (1986a) reported that the blue-green colour was due to a pH shift from 3·3 to approximately 5·0 during the degradation of malate. The wild-type and transformed strains degraded more than 99·7% of the malic acid present, whereas the mutant strain was unable to utilize malic acid.

Chromosomal localization of the mae2 *gene and size of mRNA transcript*

Osothsilp (1987) mapped the *mae2* gene 29 map units from the *ade6* gene on chromosome III of *S. pombe*. Southern analysis of the CHEF gel confirmed that *mae2* is located on chromosome III (Figure 2). The *mae2* gene encodes a single mRNA transcript of approximately 2·0 kb (Figure 3), which is in the range of 1·674 kb for duck liver malic enzyme (Hsu *et al.*, 1992) to 2·264 kb for the *Ascaris suum* malic enzyme (Kulkarni *et al.*, 1993).

Nucleotide sequence of the mae2 *gene*

A restriction map of the *mae2* gene is shown in Figure 4. The 4·6-kb *Hin*dIII fragment contains an open reading frame (ORF) of 1695 bp, with the ATG at nucleotide (nt) 2184 and the stop codon at nt 3879. The DNA sequence of the ORF and 3′-flanking region, as well as the inferred amino acid sequence, are shown in Figure 5. The 5′-flanking sequences of *mae2* contain several TATA-like sequences (data not shown).

The genetic structure of *S. pombe* differs in many aspects from that of *S. cerevisiae*, e.g. codon usage, organization of ribosomal RNA genes, presence or absence of introns, intron splicing recognition sequences, and polyadenylation signal sequences (Barker *et al.*, 1987; Nischt *et al.*, 1986; Riva *et al.*, 1982; Simon-Becam *et al.*, 1978). The intron and splicing apparatus and use of transcriptional and translational signals of *S. pombe* display a closer resemblance with that of mammalian cells than with *S. cerevisiae* (Jones *et al.*, 1988; Kaüfer *et al.*, 1985; Mertins and Gallwitz, 1987).

The presence of a poly(A) tail in eukaryotic mRNA increases the cytoplasmic stability of mRNA (Bernstein and Ross, 1989) and enhances the efficiency of its translation (Munroe and Jacobson, 1990). By using different poly(A) sites on the primary transcript, the size and levels of particular mRNAs can be varied (Smith *et al.*, 1989) as is the case for the murine malic enzyme gene (Bagchi *et al.*, 1987) and rat malic enzyme gene (Morioka *et al.*, 1989). Three signal motifs comprise a functional poly(A) site on mRNAs in mammalian cells (Manley, 1988): a highly conserved AAUAAA occurs upstream of the poly(A) site, a CA or UA usually forms the cleavage site, and a more divergent G/U- or U-rich element is located downstream. The conserved AATAAA

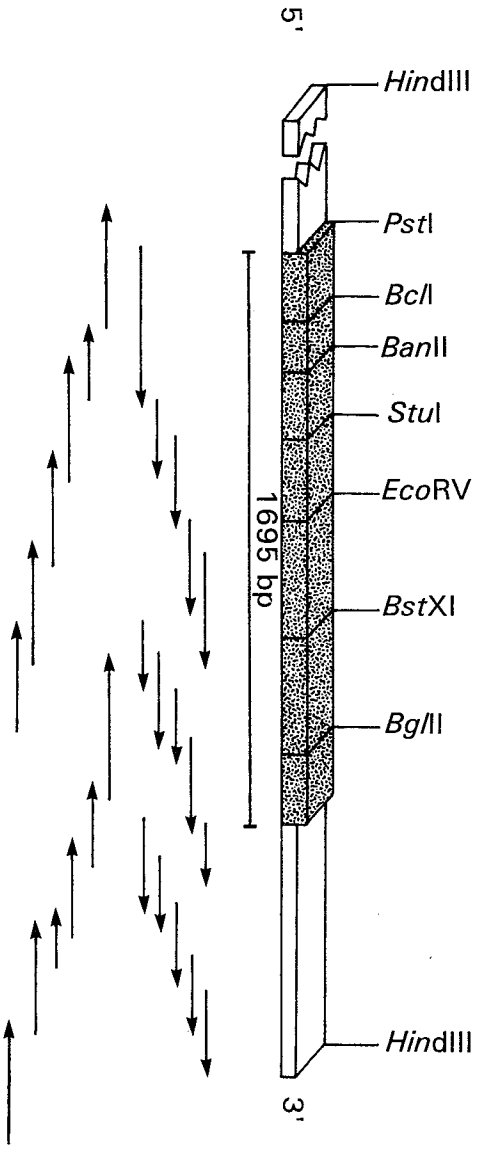

Figure 4. Restriction map and DNA sequencing strategy for the coding and 3′ region of *mae2*. Only unique restriction sites that occur within the *mae2* gene, as well as the *Ban*II site, are shown. Overlapping exonuclease fragments were generated to sequence the 4·6-kb *Hin*dIII fragment containing the *mae2* gene. Arrows indicate the regions that were sequenced on the overlapping fragments. Both strands were sequenced entirely.

sequence was found 318 bp, 344 bp, 406 bp and 489 bp downstream of the *mae2* gene. The AUUAAA motif, present in approximately 10% of eukaryotic mRNAs (Wickens and Stephenson, 1984), is present 260 bp and 494 bp downstream of *mae2*, overlapping with the AATAAA motif located 489 bp 3′-flanking to the *mae2* gene. T-rich areas are present downstream of the AATAAA motif (406 bp) and the ATTAAA motif (206 bp).

The AATAAA motif found in mammalian genes plays a small role, if any, in the polyadenylation of *S. cerevisiae* mRNA (Hyman *et al.*, 1991). Instead, *S. cerevisiae* uses a TAG . . . TATGTA or a TATATA motif at a variable distance (14 to 1240 nt) upstream of the poly(A) site (Abe *et al.*, 1990; Russo *et al.*, 1991). Humphrey *et al.* (1991) reported that the mechanism for mRNA 3′-end formation in *S. pombe* is closely related to that of *S. cerevisiae*. The *S. cerevisiae* motifs are also present in the 3′-flanking sequences of *mae2*. TATATA motifs were found 253 bp and 444 bp downstream of *mae2*, whilst a TATGTA motif is located 380 bp downstream of *mae2*.

Comparison of amino acid sequences of 13 different malic enzymes

The molecular structures of the malic enzymes of human mitochondria, mouse, rat, duck liver, *Zea maize, Populus deltoides, Flaveria trinervia, Flaveria linearis, Mesembryanthemum crystallinum* and *A. suum* (Table 1) have been reported. Comparison of the *S. pombe mae2* with these malic enzyme genes showed a DNA identity as high as 52%. There is also a high amount of identity on the amino acid level, e.g. 36·6% over 555 amino acids for the human malic enzyme. The GCG Genbank search also revealed homology with an *E. coli* sequence, namely '*E. coli sbcA8*, a translational fusion protein of part *recA* with another gene 140 kb distant' (Mahajan *et al.*, 1990). The high amount of identity obtained with this sequence (Table 1) indicates that it may contain the coding region for *E. coli* malic enzyme. There is also a high amount of identity with the *Phaseolus vulgaris* (bean) cinnamyl-alcohol dehydrogenase (*CAD4*) gene (Walter *et al.*, 1988), later reported (Walter *et al.*, 1990) to be more likely a malic enzyme.

We compared the amino acid sequences of the different malic enzymes and found several regions of highly conserved amino acid sequences. The number of amino acids between each set of elements was also consistent (Figure 6).

Wierenga *et al.* (1986) proposed a consensus sequence of hn – G – G – – G – – – n – – n – – – – – – – – n – n which serves as a βαβ fold for the binding of ADP-moieties. It has a characteristic arrangement of glycines (G), non-polar (n) and hydrophilic (h) residues at certain crucial positions on a compact βαβ fold. According to Loeber *et al.*

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1    ATGCCTGCAGGAACCAAAGAACAAATCGAGTGTCCTTTAAAAGGAGTAACTTTGTTAAACTCTCCTCGCTACAATAAGGACACTGCTTTTACACCTGAGG
     M  P  A  G  T  K  E  Q  I  E  C  P  L  K  G  V  T  L  L  N  S  P  R  Y  N  K  D  T  A  F  T  P  E  E   34

101  AGCGTCAAAAATTTGAGATTTCATCACGTCTTCCCCCCATTGTTGAAACTTTGCAACAACAAGTGGATCGCTGTTATGACCAGTACAAAGCAATCGGTGA
       R  Q  K  F  E  I  S  S  R  L  P  P  I  V  E  T  L  Q  Q  Q  V  D  R  C  Y  D  Q  Y  K  A  I  G  D   67

201  TGAGCCCTTACAGAAGAATTTGTATCTTTCTCAATTAAGCGTCACCAACCAAACTCTGTTTTACGCACTCATCAGCCAACATTTGATCGAAATGATTCCT
       E  P  L  Q  K  N  L  Y  L  S  Q  L  S  V  T  N  Q  T  L  F  Y  A  L  I  S  Q  H  L  I  E  M  I  P  100

301  ATCATCTATACACCTACCGAAGGCGATGCCATCAAGCAGTTTTCCGATATATATCGTTATCCTGAGGGTTGTTATTTGGATATTGATCATAACGATTTGT
       I  I  Y  T  P  T  E  G  D  A  I  K  Q  F  S  D  I  Y  R  Y  P  E  G  C  Y  L  D  I  D  H  N  D  L  S  134

401  CTTATATCAAGCAACAGCTTTCCGAGTTTGGAAAATCCGATAGTGTCGAATACATTATCATTACCGATTCTGAAGGTATTTTGGGTATCGGCGATCAAGG
       Y  I  K  Q  Q  L  S  E  F  G  K  S  D  S  V  E  Y  I  I  I  T  D  S  E  G  I  L  G  I  G  D  Q  G  167

501  TGTTGGTGGTGTCTTAATTTCAGTTGCCAAGGGACATTTAATGACTTTATGCGCGGGTTTAGACCCTAATCGATTCTTGCCCATTGTTCTCGATGTTGGC
       V  G  G  V  L  I  S  V  A  K  G  H  L  M  T  L  C  A  G  L  D  P  N  R  F  L  P  I  V  L  D  V  G  200

601  ACCAACAATGAAACCCATCGTAAAAATCATCAATACATGGGTTTGAGAAAGGATCGTGTTCGTGGTGAACAGTATGACAGCTTTTTGGACAATGTTATAA
       T  N  N  E  T  H  R  K  N  H  Q  Y  M  G  L  R  K  D  R  V  R  G  E  Q  Y  D  S  F  L  D  N  V  I  K  234

701  AGGCCATTCGTGAAGTCTTTCCTGAGGCCTTTATTCATTTTGAGGATTTTGGTCTTGCCAACGCCAAGCGCATTTTAGACCACTATCGTCCTGACATTGC
       A  I  R  E  V  F  P  E  A  F  I  H  F  E  D  F  G  L  A  N  A  K  R  I  L  D  H  Y  R  P  D  I  A  267

801  CTGCTTTAACGATGATATCCAGGGAACCGGTGCCGTAGCATTGGCCGCCATTATTGGCGCCCTTCACGTTACGAAATCTCCCTTAACCGAGCAGCGCATC
       C  F  N  D  D  I  Q  G  T  G  A  V  A  L  A  A  I  I  G  A  L  H  V  T  K  S  P  L  T  E  Q  R  I   300

901  ATGATCTTTGGTGCAGGTACTGCTGGTGTTGGTATCGCCAACCAAATTGTTGCCGGTATGGTGACAGATGGCCTTTCATTAGATAAGGCTAGAGGTAATC
       M  I  F  G  A  G  T  A  G  V  G  I  A  N  Q  I  V  A  G  M  V  T  D  G  L  S  L  D  K  A  R  G  N  L  334

1001 TTTTCATGATTGATCGTTGCGGTTTGCTTTTGGAGAGACATGCTAAGATTGCTACTGATGGACAAAAGCCATTTTTGAAGAAGGACTCTGACTTTAAGGA
       F  M  I  D  R  C  G  L  L  L  E  R  H  A  K  I  A  T  D  G  Q  K  P  F  L  K  K  D  S  D  F  K  E  367

1101 AGTCCCTTCTGGAGACATTAATTTAGAGAGTGCTATTGCACTCGTCAAGCCCACCATTCTTTTGGGATGTTCCGGTCAACCGGGTAAATTTACAGAGAAA
       V  P  S  G  D  I  N  L  E  S  A  I  A  L  V  K  P  T  I  L  L  G  C  S  G  Q  P  G  K  F  T  E  K   400

1201 GCCATTCGTGAAATGAGCAAGCACGTCGAGCGCCCCATCATTTTCCCAATCTCTAATCCCACTACTCTTATGGAAGCGAAGCCCGATCAAATTGACAAAT
       A  I  R  E  M  S  K  H  V  E  R  P  I  I  F  P  I  S  N  P  T  T  L  M  E  A  K  P  D  Q  I  D  K  W  434

1301 GGTCAGATGGAAAGGCTTTGATAGCCACTGGTTCCCCACTTCCTCCTCTCAATCGCAATGGTAAAAAATACGTGATTTCCCAATGCAACAATGCCCTCCT
       S  D  G  K  A  L  I  A  T  G  S  P  L  P  P  L  N  R  N  G  K  K  Y  V  I  S  Q  C  N  N  A  L  L  467

1401 TTACCCTGCTCTTGGTGTTGCATGTGTGTTATCCCGTTGCAAGTTATTGAGTGATGGTATGCTGAAAGCAGCTTCCGATGCTTTGGCCACTGTTCCCAGA
       Y  P  A  L  G  V  A  C  V  L  S  R  C  K  L  L  S  D  G  M  L  K  A  A  S  D  A  L  A  T  V  P  R   500

1501 TCTTTATTTGCTGCTGATGAAGCCCTCTTGCCAGATTTGAACAATGCTCGCGAAATTTCTCGTCACATTGTTTTTGCAGTCTTGAAGCAAGCTGTTTCTG
       S  L  F  A  A  D  E  A  L  L  P  D  L  N  N  A  R  E  I  S  R  H  I  V  F  A  V  L  K  Q  A  V  S  E  534

1601 AGGGAATGAGCACTGTGGATTTACCCAAAGATGATGCTAAATTGAAGGAATGGATTATTGAACGTGAATGGAATCCCGAATACAAGCCTTTTGTATAAAG
       G  M  S  T  V  D  L  P  K  D  D  A  K  L  K  E  W  I  I  E  R  E  W  N  P  E  Y  K  P  F  V  *      565

1701 CCTTTTATTTTATTTTTTTTTGAAACCTGCTTTTTGGTCTGCTTGTATTTAAAGATATTCATGTAAATAATTTTTTGAAAGATGAATTTACAATAAGTTG
1801 CTAAAAAGAAAATTCCCGTTTTATTCAAATGCTCATATTTGAATATTAGAAACATTATGTACATATTTAGGCATCTTCCATTAAGAATGATTAATGCGTA
1901 GAAAGATAATCAATTATTATTGCTTTTTTCTCCTATTGTTATTCATCAACTATATACATTAAAAAGATTGGAGTATAGCAGAGGTAGAATTTCTTTACTC
2001 TGAAAAGTAAATCGAAATAAATGGTATATGATTCAGTCTGAAATAAATTGAGCACGAGTATTCAAACCGTAAACCGTTATGTATTGAATGAACCATTTGA
2101 TTTAATAAAGGTTATAATTTTACGAATTTATAATGGGTAGTTATATAGAAACACCAAGTTAACTTTATAATCAGATTAATCTGAATAATAAATTAAAAAG
2201 GGAAAGAGAAATCTGTATATGGATGAAACAAACAAATAGTAAATCGCATTTGACACCTACAAAATGTGTGTGAATATATACATACAAGGAGGGCCTGTAA
2301 ATAGAACTTTGTATTCCCAAGGGATTTAGTGAACACCCTTAAAATCGTTATTACTAAATTTCGTAGATCAGTTTCTTGAAGGTAAACTCATCACCCCCAA
2401 GTCTGGCTATGCAGAAATCCCC
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Figure 5. DNA and amino acid sequence of the coding and 3′-flanking region of *mae2*. The deduced amino acid sequence of the protein is written below the nucleotide sequence. The numbers on the left refer to the nucleotide sequence, and those on the right refer to the protein sequence.

Table 1. DNA and amino acid homology of the *S. pombe* malic enzyme with other known malic enzymes

Source	DNA % identity (bp)	Amino acid % identity (aa)	EC number‡	GCG Accession code§	Reference
Human mitochondria	52·4 (1532)*	36·6 (555)†	40	M55905	Loeber *et al.* (1991)
Murine ME	51·9 (958)	35·0 (554)	40	J02652	Bagchi *et al.* (1987)
Rat			40	M30596	Magnuson *et al.* (1986)
Duck liver		33·5 (555)	40	S38982	Hsu *et al.* (1992)
Zea maize	52·9 (991)	35·0 (552)	40	J05130	Rothermel and Nelson (1989)
P. vulgaris	52·1 (1479)	33·5 (553)	40	J03825	Walter *et al.* (1988)
P. deltoides		34·1 (552)	40	X56233	Van Doorsselaere *et al.* (1991)
F. trinervia	50·9 (1033)	33·8 (554)	40	X57142	Börsch and Westhoff (1990)
F. linearis		33·1 (356)	40	M59415	Rajeevan *et al.* (1991)
A. suum		34·8 (561)	38	M81055	Kulkarni *et al.* (1993)
E. coli		41·0 (442)	38/40¶	X55956	Mahajan *et al.* (1990)
Mesembryanthemum			40	S44544	Cushman (1992)

*52·4% identity over 1532 bp, etc.
†36·6% identity over 555 amino acids, etc.
‡EC 1.1.1.38/39/40, classified according to cofactor specificity.
§GCG Genbank accession code.
¶Uncertain whether it represents the NAD- or NADP-linked enzyme.

(1991), NAD-binding sites display the consensus G – G – – G – – – G – – – – – – G – – – – – – an – – – – – n – – (n, neutral; b, basic; a, acidic), whereas the NADP-binding sites have the sequence G – G – – A – – – A – – – – – – G – – – – – – nb – – – – – b – – (Hanukoglou and Gutfinger, 1989; Scrutton *et al.*, 1990). Replacement of the two alanine residues (underlined above) with glycine residues converted the NADP-specificity of a glutathione reductase to a NAD-specificity (Scrutton *et al.*, 1990).

A consensus LGLGDLG sequence was reported for the NAD-binding site in malic enzymes (Hsu *et al.*, 1992; Wierenga *et al.*, 1986), in agreement with the X – Y – Gly – X – Gly – Y – X – Gly (X is a hydrophobic residue, Y is variable, and the indicated spacing of the three Gly residues is retained) reported for different NAD-linked dehydrogenases (Rossman *et al.*, 1975). The present study, however, revealed an expanded consensus sequence of ILGLGD – G – – G for the NAD-binding sites in malic enzymes (region B in Figure 6). However, *S. pombe* has an isoleucine instead of the second leucine residue.

A GAGEAGTGIA consensus for a NADP-binding site in malic enzymes was reported by Rothermel and Nelson (1989) and Börsch and Westhoff (1990). The sequences of maize, *P. vulgaris, P. deltoides, F. trinervia* and *Mesembryanthemum* display a GAGEAGTGIA sequence for region E (Figure 6), whereas *S. pombe* and *E. coli* have a closely related sequence (GAG – AG – GIA). The human, murine, rat and duck malic enzymes have the consensus GAGEAALGIA, in agreement with the proposed NADP site of Scrutton *et al.* (1990). The sequences of *A. suum* and *F. linearis* do not fit either of the reported consensus sequences, but possess some characteristics of both. All the malic enzymes reported in Figure 6, except *F. linearis*, have the GAG – A – – GIA sequence for region E. Furthermore, all the EC 1.1.1.40 malic enzymes (except *F. linearis*) display the consensus GAGEA – – GIA. The consensus glutamate residue (E) might therefore be important for the coenzyme specificity of the malic enzyme. However, it is difficult to designate a physiological importance to the presence of the glutamate residue in the NADP-linked enzymes without further investigation. It is clear that the consensus sequences for the NAD- and NADP-binding sites suggested by Scrutton *et al.* (1990) do not apply for these sites in malic enzymes described to date.

For region A, a consensus amino acid sequence of P – VYTPTVG – AC was found, with slight variations for *S. pombe, Mesembryanthemum* and *E. coli.* This region does not show significant identity with any regions in proteins other than the

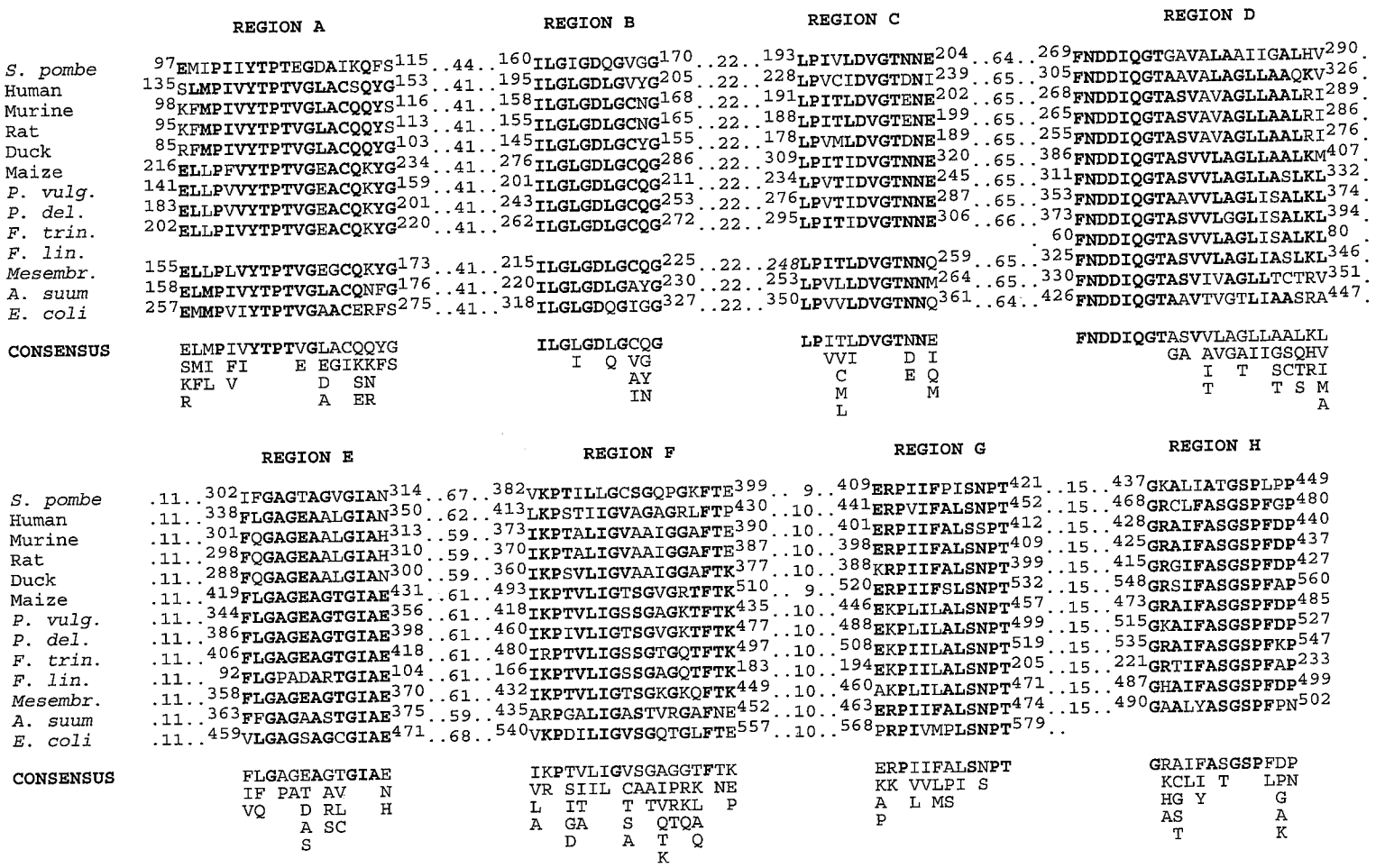

Figure 6. Regions of conserved amino acid sequences observed in the different malic enzymes. The numbers of amino acids between the regions are indicated. Bold letters indicate conserved residues and replacements are listed below the consensus sequence. The amino acid positions given here are as they appear in GCG databank, and do not therefore necessarily correspond with those published. Abbreviations used are *P. vulg. (P. vulgaris), P. del. (P. deltoides), F. trin. (F. trinervia), F. lin. (F. linearis), Mesembr. (Mesembryanthemum), B. stearo. (B. stearothermophilus).*

malic enzymes in Table 1. Rothermel and Nelson (1989) reported a VYTPTVGEA sequence at position 221–129 on the maize malic enzyme, that showed homology with the proposed NADP-binding site (VPTTVGSA) of the enoyl reductase domain of goose fatty acid synthetase, and also had some identity with the VPTTMEKA region of human glyceraldehyde-3-phosphate dehydrogenase, an NADP-dependent enzyme.

Hsu *et al.* (1992) reported that 'Cys 99' in duck liver malic enzyme (corresponding to *S. pombe's* position 111 in region A) was important for the binding of L-malate and divalent metal ions. Modification of a thiol group at the malate-binding site of the NAD-linked malic enzyme from *A. suum* resulted in a decreased binding of malate (Gavva *et al.*, 1991). Drincovich *et al.* (1992) found evidence for the presence of two sulfhydryl groups, at positions 230 and 284, located at or near the active site of the maize malic enzyme. The cysteine at position 230 corresponds to position 111 (therefore the Cys 99 in duck liver) of the *S. pombe* amino acid sequence. This cysteine is conserved in all of the malic enzymes in Figure 6, except for *S. pombe*, which contains an isoleucine instead of the cysteine residue. The other cysteine, found at position 284 in the maize malic enzyme, is present in all the NADP-dependent malic enzymes (Figure 6, region B).

Hsu *et al.* (1992) suggested that Cys 99 is involved in binding of malate and divalent ions in duck liver malic enzyme, whilst Drincovich and Andreo (1992) reported this cysteine to be adjacent to the NADP-binding site in maize malic enzyme. It therefore seems that there is uncertainty whether the sequence of region A constitutes an NADP site or the malate-binding site. However, the order of events, as reported by Pry and Hsu (1980) for pigeon liver malic enzyme, could explain this contradiction. They found that Mn^{2+} binds first, followed by NAD(P) and malate. Cys 99 could be in contact distance from the bound divalent metal ion (Schimerlik and Cleland, 1977) and could therefore play a role in the formation of a Cys-Mn^{2+} ligand. Kiick *et al.* (1984) found that the sulphydryl group in *A. suum* is not essential for enzyme activity, but the reactivity of the thiol group is modulated by the divalent metal ion. The metal ion presumably polarizes the carboxyl group of the oxalacetate intermediate during decarboxylation (Gavva *et al.*, 1991), in agreement with the accepted theory for metal-assisted decarboxylation of β-keto carboxylic acids (Grissom and Cleland, 1988). The Cys-Mn^{2+} ligand therefore seems to play an important role in the catalytic mechanism; however, the absence of a 'Cys 99' in *S. pombe* malic enzyme questions the requirement for this residue.

No significant homology with amino acid sequences in the Genbank databank was found for regions C, D, F, G and H, and the physiological importance of these regions remains unknown. However, the fact that these conserved regions were found consistently in the malic enzymes from bacteria, fungi, nematodes, higher plants, animals and humans, suggests that these regions are indispensable for the expression of malic enzyme activity.

ACKNOWLEDGEMENTS

We thank J. Grobler for his assistance with the GCG program and members of the Stellenbosch Yeast Group who read the manuscript and offered suggestions for its improvement. This work was funded by FRD grant 2016111 and a grant from the South African Wine Industry to H. J. J. van Vuuren.

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