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Mutational analysis of malate pathways in *Schizosaccharomyces pombe*

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Schizosaccharomyces pombe is of interest to wine-makers because, unlike the *Saccharomyces cerevisiae* wine yeasts, it efficiently metabolizes all levels of malic acid found in grape musts. To determine the metabolic pathway for malate metabolism in *Sch. pombe*, a mutational analysis was performed using a selection system which isolated 154 mutants that were unable to utilize malic acid. The mutants were in three complementation groups which corresponded to alleles and activities for malate dehydrogenase, malic enzyme and malate transport respectively. Under anaerobic conditions *Sch. pombe* ferments malic acid by using a malate transport, malic enzyme, pyruvate decarboxylase, alcohol dehydrogenase pathway (a malo-ethanolic fermentation) but under aerobic conditions malate is both fermented to ethanol and to a much lesser extent, respired as a TCA cycle intermediate. Alleles were mapped genetically and the locations were confirmed by chromoblotting. Malic enzyme and malate transport alleles were cloned by complementation and the proteins have been purified and partially characterized. © 1998 Canadian Institute of Food Science and Technology. Published by Elsevier Science Ltd. All rights reserved

INTRODUCTION

Excess malic acid in grape musts is a concern to wine-makers dealing with vines that have been over-cropped or if the growing season has been particularly cool and/or wet. The resulting wines can taste very sour. Additionally, small amounts of malic acid in bottled wines can support the growth of lactic acid bacteria which can spoil the wine through a malo-lactic fermentation. Reduction and control of malic acid concentrations in wines has been the subject of several reviews (Radler, 1975, 1993; Kunkee, 1991; Hennick-Kling, 1993) and publications (Spettoli *et al*., 1987; Edwards and Beelman, 1989; Gao and Fleet, 1994).

The ability of certain yeasts to metabolize malic acid has been known for some time (Kluyver, 1914). Depending on the strain, *Saccharomyces* spp wine yeasts will ferment 0 to 3 g l^{-1} malate during vinification. Malic enzyme in Saccharomyces has a low (km = 50 mM) substrate affinity (Fuck and Radler, 1972) so most malate is anabolically utilized through malate dehydrogenase and the oxidative reactions of the tricarboxylic acid (TCA) cycle (Salmon *et al*., 1987)

Sch. pombe can ferment up to 29 g malic acid per liter of medium (Taillandier *et al*., 1988; Taillandier and Strehaiano, 1991). Our eventual objective is to construct a Saccharomyces wine yeast with the capacity to utilize 7 g l^{-1} malic acid. This requires a mutational analysis of malate utilization in *Sch. pombe* in order to determine the quintessential differences in malate metabolism between Saccharomyces and Schizosaccharomyces. We have previously characterized the malic enzyme (Osothsilp and Subden, 1986*a*) and malate transport mutants (Osothsilp and Subden, 1986*b*) and the gene sequence data elsewhere (Viljoen *et al*., 1994; Grobler *et al*., 1995). The present work reports the malate dehydrogenase data plus genetic and pathway studies for malate utilization in *Sch. pombe*.

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MATERIALS AND METHODS

Microorganisms, culture media and mutagenesis

Wild-type strains of *Sch. pombe* 972 h^- originated from the collection of Leupold, (1950) and strains with meiosis defective mating type allele "mat 2-B102" (Gygax and Thuriaux, 1984) were kindly donated by P. Thuriaux (Inst. für allgmeine Mekrobiologie, University of Bern, Bern, Switzerland). For the linkage studies, strains carrying the markers: *lys2-97 h⁻, lys3-37 h⁻, leu2-120 h⁻, his1-1 h⁺, met1-1 h⁻, met2-1 h⁻, ade2-17 h⁻, ura2-10, ade6-260 h⁻, arg1-320, ade7-1143 h⁻* and *rad1-1 h⁻* were obtained from A Nassim (National Research Council of Canada, Ottawa), *ura1-161 h⁻, ade3-58 h⁻, his6-365 h⁻, pro2-1 h⁻*, and *ade4-31 h⁻* were obtained from P.Young of the Biology Dept., Queen's University (Kingston, Ontario), and *ura4-294 h⁻, tyr1-62 h⁻, ade5-26 h⁻, trp3-25 h⁺*, and *lys1-131 h⁻*, were obtained from P. Munz, Bern University, (Bern, Switzerland). For the gene rescue experiments, we constructed recipient strains using a *leu1-32* selective marker (from A. Nassim), which complements *LEU2* from Saccharomyces carried on plasmid pWH5 (from P. Young).

Yeast extract media (YE and YEA), synthetic sporulation agar (SPA), and modified Wickerham agar MML (10 g glucose, 10 g L-malic acid, 5 g $(NH_4)_2SO_4$, 1 g KH_2PO_4, 0.5 g $MgSO_4$. $7H_2O$, 0.1 g NaCl, 0.1 g $CaCl_2.2H_2O$, 10 mg meso-inositol, 10 mg nicotinic acid, 1 mg Ca pantothenate, 500 µg H_3BO_3, 400 µg $ZnSO_4.7H_2O$, 400 µg $MnSO_4.H_2O$, 160 µg $H_2MoO_4.2H_2O$, 200 µg $FeCl_3.6H_2O$, 100 µg Kl, 40 µg $CuSO_4.5H_2O$, and 10 µg biotin in 1 l of distilled water) were described by Leupold, (1950), Leupold, (1955). Malate-glucose indicator media (MGI and MGIA) for mutant isolation were developed in this laboratory. The MGI medium is composed of 10 g l-malic acid, and 0.1 g brom cresol green in 1 L of MML. The pH of the medium is adjusted to 3.3 with KOH. For MGIA medium, 15 g l^{-1} of Bacto agar is added into MGI broth.

Sch. pombe was cultured in 1 l side arm flasks containing 200 ml of YE broth. The flasks were shaken on a gyrotory shaker at 150–200 rpm at 28°C. Anaerobic cultures were prepared using 300 ml of medium in a 500-ml side-arm flask equipped with a vapour lock containing 0.02% (w/v) sodium azide and were incubated at 28°C without shaking. Cell concentrations were estimated using OD at 600 nm and compared to a standard curve.

Cells of the haploid *Sch. pombe* 972 h^- from a 24 h culture in YE broth at 30°C were centrifuged and washed twice with 0.2 M acetate buffer (pH 5) and suspended in the same buffer. One milliliter of cell suspension adjusted with 0.2 M acetate buffer (pH 5) to a concentration of approximately 4.5×107 cells/ml^{-1} was treated at 30°C with 2 ml of a 6 mg/ml^{-1} solution of *N*-methyl-*N*′-nitro-nitrosoguanidine (NTG) as described by Magnet, (1965). The solution was prepared just prior to the start of the experiment. The survival curve was prepared by exposing the cells to NTG for various times then plating the appropriate dilution (in acetate buffer) on MGIA. An exposure time resulting in a 10–30% survival was selected for the mutagenesis experiments. Mutants unable to utilize malate had cream colonies and the wild type were blue on the MGIA plates.

Complementation test

Complementation analysis of various malic enzyme deficient mutants was performed according to the method described by Gutz *et al.*, (1974). Two mutant strains (*972h⁻* and *mat 2-B102*), each carrying auxotrophic markers (*leu 1-32* and *lys 1-131*, respectively) were crossed on SPA plates and incubated at 25°C for 13–15 h. The diploid zygotes were then transferred onto indicator agar plates (MGIA) without amino acids. The diploid nature of the prototrophs grown on the indicator agar plates was confirmed by streaking onto a YEA plate containing phloxin B dye (Kohli *et al.*, 1977). Diploid colonies were red, while haploid ones were pale pink on such plates. Diploid cells also have a greater length and diameter at cell division (Nurse and Thuriaux, 1980).

Analysis of metabolic intermediates

A high performance liquid chromatographic system (Waters Associates Inc. Milford, MA) was used with a M6000-A solvent delivery unit, and a U6K universal liquid chromatography injector coupled through a column to an R401 differential refractometer. The organic acid analysis column model HPX-87H was obtained from Bio-Rad (Berlin, Germany) Samples from 6 day old wild type and mutant cultures grown in MML medium were filtered through 0.45 mm metricel filter membranes and degassed before injection. The solvent was 0.01 N H_2SO_4 with the flow rate adjusted to 0.5 ml min^{-1}. The chromatograms were obtained from a Fisher Recordall series 5000 recorder with a chart speed of 0.25 cm min^{-1}. The critical peaks were glucose, tartaric acid, malic acid and ethanol. The fermentation was monitored by noting the conversion of glucose into ethanol which occurred in all strains. Tartaric acid remains unaffected by fermentation. The malic acid peak disappeared in the medium of wild types but persisted in the medium of malic acid utilizing mutants. To confirm the identity of the mutant malic acid utilizing strains, analyses were repeated in triplicate.

Preparation of cell-free extracts

The washed cells of each culture were suspended in 0.05 M tris(hydroxymethyl) amino-methane hydrochloride buffer (pH 7.8) on a 1:1 volume ratio and were transferred to a 10-ml glass vessel containing glass beads

(diameter, 0.45 mm). The weight ratio of glass beads to cell suspension was 3:1. The cells were cooled on ice and then were disrupted with a mechanical cell homogenizer (Mickle Laboratory Engineering Co., U.K.) for 6 min at 6000 cycles min^{-1}. Disruption of cells was confirmed microscopically. At the above speed and time, more than 50% of the cells were disrupted. The glass beads were removed from the homogenate by filtration through a cotton-plugged pipette tip. Insoluble debris and undisrupted cells were removed by centrifugation at 40 000 g at 4°C for 20 min. The supernatant fluid was used directly for enzymatic analyses by spectrophotometry and starch-gel electrophoresis.

Enzyme assays

Activities of malic enzyme were modifications of the method of Zink, (1990). The assay mixture consisted of 0.1 ml of 0.2 M l-malate, 0.05 ml of 4×10^{-3} M NAD or NADP, 0.05 ml of $MgCl_2$ or $MnCl_2$, 0.1 M phosphate buffer (pH 7.5), and enzyme solution to make 3 ml. A 0.1 M glycine-NaOH buffer pH 10 was substituted when the forward reaction of MDH was measured. The reaction was started by adding the enzyme preparation. The increase in absorbance at 340 nm was measured on a Zeiss (Jena, Germany) recording spectrophotometer DMR 21. The relative rate of reaction was expressed as absorbance change per minute. The activity of malic enzyme was also confirmed by determining the appearance of pyruvic acid on the high-pressure liquid chromatogram. Assays for malate transport protein are given elsewhere (Osothsilp and Subden, 1986*b*).

Starch-gel electrophoresis

The electrophoresis was run in a horizontal slab gel. The gel consisted of 12% (w/v) starch and 10% (w/v) sucrose, in 1:20 dilutions of electrode buffer. The electrode buffer was morpholine-citrate (pH 6.1), originated from Clayton and Tretiak, (1972). Electrophoresis was performed at a regulated voltage of 300 V/14 cm gel for 6–12 h at 4°C. After electrophoresis, the gel slab was stained to visualize the enzymes. The staining procedure was based on that of Shaw and Siciliano, (1976) for malic enzyme (ME) and malate dehydrogenase (MDH).

Mapping strategy

Genetic mapping of mau genes followed the method described by Gygax and Thuriaux, (1984) with some modifications: (i) A strain carrying the marker being considered and *leu1* was crossed to a *lys1* mutant in order to construct the h^- triple mutant (*mau leu1 lys1*) needed for step 2. (ii) The h^- triple mutant was crossed to a *mat2- B102, ade6-704* strain, and the resulting diploids are selected by complementation on a medium lacking lysine, leucine, and adenine. After haploidization by m-fluorophenylalanine, about 100 haploid clones were identified by their pale colony pigmentation in the presence of phloxine B and by their cell length at division. These haploids were analyzed for the cosegregation of the marker under study with *lys1* (chromosome I), *leu2* or *mat 2-B102* (chromosome II) or *ade6* (chromosome III). *lys1, leu2* and *ade6* were chosen as tester markers because, in contrast to many auxotrophic markers, they are not counter-selected during haploidization (Kohli *et al*., (1977)) whereas their strong linkage with the centromere minimizes the chance of a mitotic crossover which would obscure the interpretation of the segregation data. (iii) Once the marker had been allocated to one of the three chromosomes, the strain was crossed to a set of appropriate tester strains carrying markers on that chromosome. About 400–1000 spores were collected after selective killing of the vegetative cells and the resulting colonies were analyzed for the segregation of the different markers and for the linkage of the tester markers with the marker under study.

Chromoblotting

Pulse field contour clamped homogeneous electric field (CHEF) electrophoresis and chromoblotting were performed by the procedure described by Van der Westhuizen and Pretorius, (1992). *Sch. pombe* 972 (wild type) was grown in 100 ml YEPD until the late logarithmic phase. Chromosomal DNA plugs were prepared and loaded into a 0.75% agarose (Pulsed Field Certified, Bio-Rad Laboratories, Hercules, CA) electrophoretic gel, and run in 0.5X TE buffer for 150 h at 50 V and 14°C, with a switching time of 60 min.

The gel was prepared for Southern blotting by depurination for 2 ×15 min with 0.25 M HCl, denaturation (0.5 M NaOH and 1.5 M NaCl) for 2 × 30 min, and neutralized (1 M Tris–Cl (pH 8), 10 mM EDTA, 1.5 M NaCl) for 2×30 min. After each step the gel was rinsed with distilled water. The DNA was transferred to a 0.45 μm Hybond-N nylon membrane (Amersham International, Buckinghamshire, UK) as described by Sambrook *et al*., (1989). The internal 610-bp EcoRV/BamII DNA fragment of *mae2* was labelled with a-^{32}P-dATP by using the random primed DNA labelling kit (Boehringer Mannheim GmbH, Mannheim, Germany) and used as a probe to detect the *mae2* gene.

Cloning

A HindIII *Sch. pombe* library in pWH5 was used to transform the malate transport defective and malic enzyme deficient mutants according to the method of Beach *et al*., (1982). Transformants were transferred to one mL of MGI medium and complementation was detected colorimetrically.

RESULTS AND DISCUSSION

Mutant isolation and characterization

The medium of MGIA plates is yellow and the wild type *Sch. pombe* colonies were yellow in the beginning but turned deep blue after three days and the mutant colonies were cream coloured. A total of 184 cream coloured colonies and a single green colony were isolated. HPLC analysis indicated that malic acid utilization in 154 of the cream coloured mutant strains, varied from 0 to 50% of the malic acid present in GMI broth. The remaining 30 other mutants were not studied further as they utilized $>50\%$ of the malate or were difficult to culture.

Diploid phenotypes were used to divide the mutants into three complementation groups. Isozyme analysis of group I mutants indicated a loss of malic enzyme activity (Osothsilp and Subden, 1986*a*), group III with loss of malate transport activity (Osothsilp and Subden, 1986*b*) and the single green group II mutant (*mdh-1-124*) with loss of MDH activity (Fig. 1). *mdh1-124* used 49% of the malate in MGI under aerobic conditions (resulting in the green colour) but had a similar phenotype to the wild type under anaerobic conditions. Malic enzyme defective mutants metabolized no malate anaerobically but could metabolize 10% of MGI malate aerobically indicating that *Sch. pombe* may metabolize malate by more than one pathway. Log and stationary phase *Sch. pombe* possessed three and four MDH isozymes, respectively. The exact role of the constitutively synthesized MDH I is not known. MDH II appeared only in aerobic cultures. *mae1-118* is defective in malic enzyme, has increased MDH II and IV activity and is able to use 34% MGI malate aerobically (other *mae1* mutants use about 18%) and 10% anaerobically (other *mae1* mutants use about 7%). Therefore, MDH II likely plays a significant role in the aerobic degradation of malate. MDH III is a glucose repressed isozyme and is unlikely to be involved with the degradation of exogenous malate since malate degradation occurred in the presence of high glucose concentrations. MDH IV appears only in stationary phase so it is an unlikely factor in the metabolism of exogenous malate. This work extends the report by Flury *et al.* (1974) who was able to isolate two MDH isozymes in *Sch. pombe*.

The *mdh1-124* lost isozymes MDH I, II and IV (Fig. 1, lanes 7 and 8). To determine whether this was a single or multiple mutation, *mdh1-124* was crossed with wild type and 80 random spores were analyzed. Thirty-six produced MDH III only and 44 possessed wild type MDH isozymes indicating cosegregation from a single allele. The *mdh1-124* allele seems to have affected the regulation of ME. The results presented are consistent with data presented by other authors (Flury *et al.*, 1974; Steffan and McAllister-Henn, 1992; Minard and McAllister-Henn, 1992; Minard and McAllister-Henn, 1994). The green colony colour is consistent with the 'verdant' respiratory deficient phenotypes described by brewers using brom cresol green based media (Green and Gray, 1950). The MDH genes were considered not to have the potential for the construction of the malate fermenting yeasts so were not considered further.

Malic enzyme defective mutants metabolized no malate anaerobically but could metabolize approximately 10% of the MGI malic acid aerobically indicating that *Sch. pombe* may metabolize malate by more than one pathway.

Mapping and chromoblotting

On the initial cross to the triple marked tester strain *mae1* was mapped to *ade6* on chromosome III and both *mdh1* and *mtp1* to *lys1* on chromosome I. Crosses between the markers shown in Fig. 2 and *mtp1* placed the *mtp1* allele 9.5 map units from *rad-1* and 33.7 map units from *ade2*. Crosses between *mtp1* and *mdh* indicated a close linkage of one map unit.

Notwithstanding some lethality associated with the ADE6 *mae1* parental class, crosses between *mae1* and the markers shown in Fig. 2 placed the *mae1* allele 29 map units distal to *ade6* on the right arm of chromosome III. The chomoblotting results (data not shown) support the mapping data for *mtp* and *mae*.

Cloning

Strains *leu1-32*, *mae1-167* and *leu1-32*, *mtp1-129* were constructed for the transformation study because they

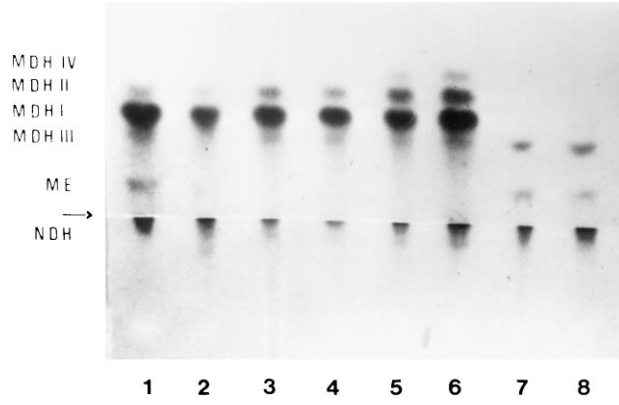

Fig. 1. Starch gel electrophoresis for the analysis of MDH isoenzymes in wild-type, *mae1-118*, *mael-167*, and *mdh1-124* strains. The yeast cells were grown in YED medium. Lanes 1 and 2 were loaded with cell-free extracts from wild-type cells containing 100 and 50 µg protein respectively. Lanes 3 and 4 were from *mael-101* and *mae1-167*, each containing 50 µg protein. Lanes 5 and 6 were 50 and 100 µg protein from *mae1-118* cell-free extract. Lanes 7 and 8 were 50 and 100 µg protein from *mdh1-124* cell-free extract. MDH activity was stained at pH 8 for 3 h. The nothing dehydrogenase (NDH) bands are believed to be alcohol dehydrogenase acting on cryptic substrate in the gel.

were stable and had low reversion frequencies. It was not possible to determine complementation from colony phenotype so each transformant was picked off and used to inoculate 1 ml of MGI broth. After 54 000 mini-fermentations a 5.6 kb fragment complementing *mae1* and a 1.6 kb fragment complementing *mtp* were cloned. The activity was confirmed by isozyme and intermediate pool analysis. *S. cerevisiae* Y99 failed to ferment malate when transformed with either or both of these genes. In a further study, in the laboratory of Van Vuuren, we have reported (Volschenk *et al*., 1997*a*,*b*), that the *mae1-167* and *mtp1-129* structural genes can be expressed if placed under the control of flanking sequences from *S. cerevisiae* and that construction of a *S. cerevisiae* strain capable of a full malo-ethanolic fermentation is possible.

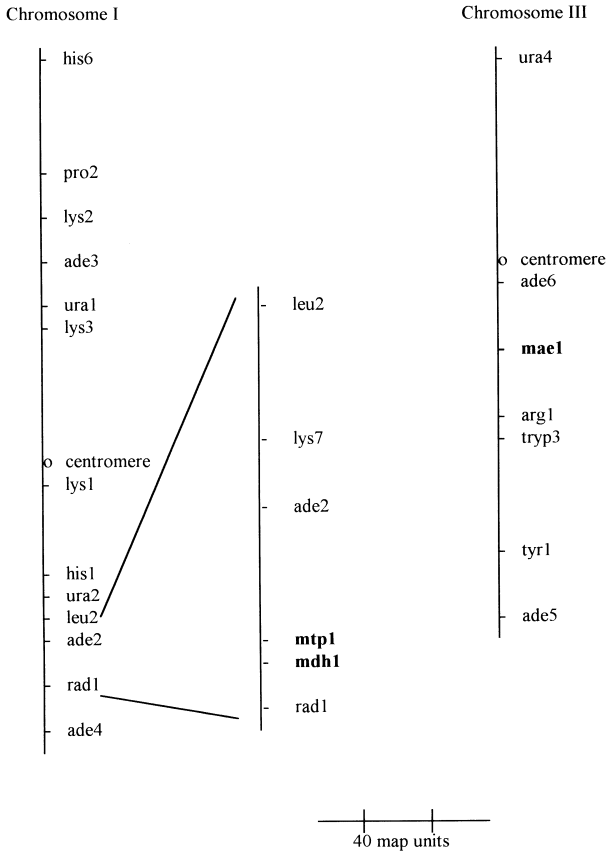

Fig. 2. Recombination map of chromosome I and III showing the markers used and the locations of mtp, mdh and mae 1.

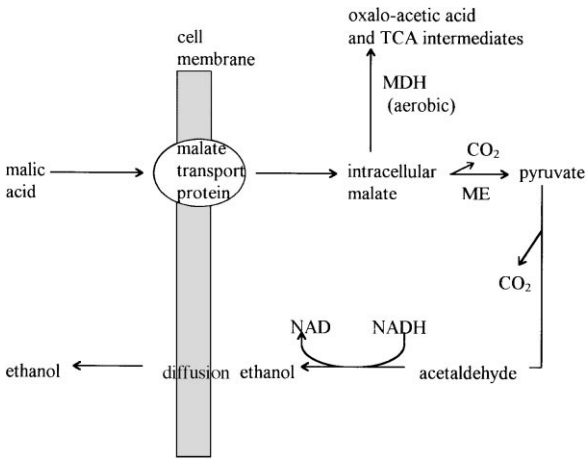

Fig. 3. Malate pathway in *Schizosaccharomyces pombe*.

Pathway studies

Isozyme analysis and high pressure liquid chromatographic monitoring the fate of malate in wild type and mutant strains of *Sch. pombe* (Osothsilp and Subden, 1986*a*,*b*) indicate a stoichiometric conversion of malate into ethanol (a malo-ethanolic fermentation) under anaerobic conditions via the pathway shown in Fig. 2.

The mutational analysis has verified a *Sch. pombe* malic acid fermenting pathway (malic acid, pyruvic acid, acetaldehyde, to ethanol) that had been previously predicted by Kuczynski and Radler, (1982).

The identification of the *Sch. pombe* malic acid transport gene holds considerable interest for research into the construction of malo-ethanolic and malo-lactic fermenting wine yeast as well as various groups investigating the transport of malic acid across various intracellular membranes in the organelles of higher plants of agricultural importance.

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