The putative monocarboxylate permeases of the yeast Saccharomyces cerevisiae do not transport monocarboxylic acids across the plasma membrane

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Abstract

We have characterized the monocarboxylate permease family of Saccharomyces cerevisiae comprising five proteins. We could not find any evidence that the monocarboxylate transporter-homologous (Mch) proteins of S. cerevisiae are involved in the uptake or secretion of monocarboxylates such as lactate, pyruvate or acetate across the plasma membrane. A yeast mutant strain deleted for all five MCH genes exhibited no growth defects on monocarboxylic acids as the sole carbon and energy sources. Moreover, the uptake and secretion rates of monocarboxylic acids were indistinguishable from the wild-type strain. Additional deletion of the JEN1 lactate transporter gene completely blocked uptake of lactate and pyruvate. However, uptake of acetate was not even affected after the additional deletion of the gene YHL008c, which had been proposed to code for an acetate transporter. The mch1–5 mutant strain showed strongly reduced biomass yields in aerobic glucose-limited chemostat cultures, pointing to the involvement of Mch transporters in mitochondrial metabolism. Indeed, intracellular localization studies indicated that at least some of the Mch proteins reside in intracellular membranes. However, pyruvate uptake into isolated mitochondria was not affected in the mch1–5 mutant strain. It is concluded that the yeast monocarboxylate transporter-homologous proteins perform other functions than do their mammalian counterparts.

Keywords: monocarboxylate transport; pyruvate; lactate; acetate; functional analysis; mitochondria; yeast; MCT genes

Introduction

Saccharomyces cerevisiae is able to utilize short-chain monocarboxylic acids as sole carbon and energy sources under aerobic conditions. Transport across the plasma membrane is the first step in the metabolism of these substrates. Two different uptake systems for monocarboxylic acids have been described in S. cerevisiae: one which is shared by acetate, propionate and formate, and another which transports lactate, pyruvate, acetate and propionate (Cássio et al., 1987; Casal et al., 1995, 1996). The first system is constitutively expressed in cells growing on non-fermentable carbon sources, while the lactate transporter is specifically induced in cells grown in lactic acid medium. Both systems are completely repressed in the presence of glucose, and in cells growing in glucose medium monocarboxylates can cross the plasma membrane only by passive diffusion of the undissociated form of the acids.

With the advent of gene-sequencing technologies, the primary structure of many genes became available, revealing the strikingly hydrophobic nature of various types of integral membrane transporters (Saier, 2000). In a first attempt to group membrane transport proteins or potential transporters of S. cerevisiae (which was done prior

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to the complete sequencing of the yeast genome), in silicio analysis revealed a family of two putative monocarboxylate transporters, the ORFs YKL221w and YNL125c/ESBP6, which exhibited transporter-like topological features and shared weak similarity to the mammalian monocarboxylate transporters (MCT1 and MCT2) and to the human X-linked PEST-containing transporter (XPCT) (André, 1995). Based on binary sequence comparison, a computer-aided analysis of the 5885 initially predicted open reading frames (ORFs) of the entire genome of S. cerevisiae identified four members of the monocarboxylate permease homologues (MCH) family, comprising the ORFs YOR306c, YOL119c, YKL221w and YNL125c, similar to mammalian monocarboxylate permeases (Nelissen et al., 1997). Another analysis, carried out by Bruno André and published in the Yeast Transport Protein database (YTPdb) (http://alize.ulb.ac.be/YTPdb/), revealed the existence of still another protein, encoded by the gene YDL054c, exhibiting striking similarities to the other Mch proteins.

The known and putative monocarboxylate transporter-related sequences from eukaryotes and prokaryotes were recently reviewed by Halestrap and Price (1999), dividing the known monocarboxylate transporters into two categories: sequences related to MCT1 and sequences not related to MCT1. Related to the MCT1 gene, nine mammalian proton-linked monocarboxylate transporters (MCTs) were found, each having a different tissue distribution. Besides the five members of the monocarboxylate porter family of S. cerevisiae and several genes from other origins, including bacterial species, six related proteins were also recognized in the nematode Caenorhabditis elegans and seven possible members were identified in Drosophila melanogaster. According to the transporter classification (TC) system, these genes comprise the monocarboxylate porter (MCP) family, No. 2.A.1.13 (Paulsen et al., 1998; Saier, 2000).

Jen1 belongs to the sequences not related to MCT1. JEN1 is required for the uptake of lactate and other monocarboxylates in S. cerevisiae (Casal et al., 1999). Probably, Jen1 is directly involved in monocarboxylate transport. However, it cannot be completely excluded in so far as JEN1 codes for a regulator of monocarboxylate transporters, instead of being a transporter itself. Alternatively, it might be that Jen1 forms heteromeric complexes with the Mch proteins, thereby constituting active monocarboxylate transporters. So far, this is the first and sole gene known to be involved in monocarboxylate transport in yeasts. Jen1 belongs to the TCfamily No. 2.A.1.12, the sialate–proton symporter (ACS) family (Paulsen et al., 1998; Halestrap and Price, 1999; Saier, 2000).

In this paper, a study is presented on the five MCH genes of S. cerevisiae encoding putative membrane transporters of monocarboxylic acids. The aim of this work was to identify possible roles of the genes as functional carriers and to investigate whether they could be correlated with the utilization of monocarboxylates. All five ORFs belonging to the monocarboxylate porter family of S. cerevisiae were disrupted in one yeast strain in order to access redundant functions in monocarboxylate transport. Two other ORFs were selected for additional deletion and analysis, viz. ORF YHL008c, which has been proposed to code for an acetate–proton symporter, as it resembles bacterial formate–nitrite transporters (Paulsen et al., 1998), and JEN1.

Materials and methods

Yeast strains and growth conditions

All yeast strains constructed in this work were derived from strain CEN.PK113-13D (MATa ura3-52 MAL2-8^{c} SUC2). For batch cultures, yeast cells were grown at 30°C on a rotary shaker in YEP medium (1% yeast extract, 2% bactopeptone), synthetic minimal (SM) medium [0.67% Difco yeast nitrogen base + $(NH_4)_2SO_4$, supplemented for auxotrophic requirements] or synthetic complete (SC) medium with different carbon sources (Zimmermann, 1975). For continuous cultures, yeast cells were grown in glucose-limited chemostats at pH 5.0 (maintained by addition of 2.0 M KOH) and 30°C in a Biostat B fermenter (B. Braun Biotech, Melsungen, Germany) on a modified CBS medium (Verduyn et al., 1992) with a working volume of 1.0 l and a dilution rate of 0.1/h. The stirrer rate was 800 rpm, with an air-flow rate of 1.5 l/min (controlled by a Brooks 150/03 mass flow controller), to give a dissolved oxygen tension of at least 70% of air saturation. Glucose concentration in the reservoir medium was 5 g/l. Cultures were checked for the occurence of oscillations by continuous registration of the dissolved oxygen concentration. The data presented refer to steady states without detectable oscillations.

Construction of multiple deletion mutants

Molecular biology techniques were performed using published procedures (Sambrook et al., 1989). Strains lacking the genes YDL054c/MCH1, YKL222w/MCH2, YNL125c/MCH3, YOL119c/MCH4, YOR306c/MCH5, YKL217w/JEN1 and YHL008cwere constructed using the loxP::kanMX::loxP/Cre recombinase system and 'short flanking homology PCR' technology (Güldener et al., 1996). The primers used for the construction of the replacement PCR constructs (obtained from MWG Biotech) are listed in Table 1. The monocarboxylate transporter-homologous genes were deleted successively in the following sequence: MCH5, MCH4, MCH3, MCH2, MCH1, JEN1 and YHL008c, selecting for G418 resistance on YEP medium with 2% glucose. The correct replacements were confirmed by PCR.

Construction of promoter–lacZ fusions

Genomic replacements of the coding regions of all MCH genes by a PCR-amplified lacZ–kanMX reporter cassette were used to fuse the promoters and the first 48 nucleotides of the truncated ORFs to the E. coli lacZ gene (Boles et al., 1998). For PCR amplification of the lacZ–kanMX reporter cassette, plasmid pUG6lacZ (Boles et al., 1998) and the primers listed in Table 1 were used. Whole yeast cell PCR was performed to confirm the correct replacements in the G418-resistant transformants.

Genomic HA-tagging of MCH3

Three consecutive copies of a HA (hemagglutinin) epitope were fused in the genome to the carboxy-terminal end of the MCH3 ORF by using a modification of the PCR targeting technique (Güldener et al., 1996). First, the two primers HATAG-1 and HATAG-2 (Table 1), which contain a 16 bp complementary sequence at their 3k ends, were used in a PCR reaction to amplify each other. The resulting 134 bp fragment was cleaved with XhoI at one end and cloned between the PvuII and SalI restriction sites in front of the first loxP site of plasmid pUG6, resulting in plasmid pUG6–HA. This plasmid was then used as a template to generate, by PCR with primers S12-MCH3 and S22-MCH3 (Table 1), a DNA molecule consisting of a 3r-HA–kanMX

Table 1. Primers used in this work

Dmch1	5k-ATGCCTCTATCAAAGGTGGAGCACTACCTTTCATACCATACGCGCTTATTCGTACGCTGCAGGTCGAC-3k
	5k-ATAAGCTATGAATTTTAAAAAAAATAAATGTAGCAGTTTCTTTTTTGTGCATAGGCCACTAGTGGATCTG-3k
Dmch2	5k-ATGTCCGAAGAACGGCATGAAGATCATCATAGGGATGTTGAAAATAAATTCGTACGCTGCAGGTCGAC-3k
	5k-GACTCTCCTAGGTAATTGTTTATAACGAAAAAGACATTTACTCATATGGCATAGGCCACTAGTGGATCTG-3k
Dmch3	5k-ATGTCAACGCACTCAAACGACTACTTTTCTGCTTCTTCCGGAATGGTCTTCGTACGCTGCAGGTCGAC-3k
	5k-GCGACGTAACTATCGCGTATATAACATGAATCAGGTCGTCGAAAAGAAGCATAGGCCACTAGTGGATCTG-3k
Dmch4	5k-ATGTTGAACATTCCCATAATTGCAAACTCCAAGAGGTTCCTGTTCTCATTCGTACGCTGCAGGTCGAC-3k
	5k-ACGAAATACCCCCCCCCCCCCCTATTGGAACGCTTCTGAACGCTGACCGCATAGGCCACTAGTGGATCTG-3k
Dmch5	5k-ATGAGCTCAGACAGTTTAACGCCTAAAGACACTATAGTTCCAGAAGAATTCGTACGCTGCAGGTCGAC-3k
	5k-ATATTATTGCATTACTTTTTTGAAGATCTATAAAGGGCACTGTCTTACGCATAGGCCACTAGTGGATCTG-3k
Djen1	5k-ATGTCGTCGTCAATTACAGATGAGAAAATATCTGGTGAACAGCAACAATTCGTACGCTGCAGGTCGAC-3k
	5k-TGAAATGCAGTTACATAGAGAAGCGAACACGCCCTAGAGAGCAATGAAGCATAGGCCACTAGTGGATCTG-3k
DYHL008c	5k-ATGGTTGACGACTCAAACTATCTTACACCACATGAAACTGCATTAGCGTT CGTACGCTGC AGGTCGAC-3k
	5k-TGATTTATTTTTTTTTGTTTTCGTTGATATCGCGGGCATCTTAACTCTTC GCATAGGCCACTAGTGGATCTG-3k
HA-TAG1	5k-GCCGGTGCTGGATCCGGTTACCCATACGACGTTCCAGACTACGCTGGTGCCTACCCTTACGATGTCCC
	TGATTACGCCTCTG GTACCGCT-3k
HA-TAG2	5k-CCCTCGAGTCATTAGGCGGCGTAGTCAGGAACATCGTATGGGTAAGCGGTACCAGAGGCG-3k
S12-MCH3	5k-GGTCCGGGCATTAAAAAATACTTCCTAAGAATGGTATATCCAATGAAGGTCGCCGGTGCTGGATCCGGT-3k
S22-MCH3	5k-GCGACGTAACTATCGCGTATATAACATGAATCAGGTCGTCGAAAAGAAGCATAGGCCACTAGTGGATCTG-3k
RGFPMCH1	5kAACAAGAATTGGGACAACTCCAGTGAAAAGTTCTTCTCCTTTACTCATAATTCTGAGTTTTCTACTTTTTAA-3k
RGFPMCH2	5kGACAACTCCAGTGAAAAGTTCTTCTCCTTTACTCATACCAGCACCAGCGACTCTCCTAGGTAATTGTTTATAACG-3k
RGFPMCH3	5kGACAACTCCAGTGAAAAGTTCTTCTCCTTTACTCATACCAGCACCAGCGACGTTCATTGGATATACCATTC-3k
RGFPMCH4	5kGACAACTCCAGTGAAAAGTTCTTCTCCTTTACTCATACCAGCACCAGCAAACTTACAAAGCTTCGCACC-3k
RGFPMCH5	5kAACAAGAATTGGGACAACTCCAGTGAAAAGTTCTTCTCCTTTACTCATAAATCTGACCCACTTGAAGCC-3k
T1-ORF	5k-GTAATACAGGGTCGTCAGATGCATAGATACAATTC TATTACCCCCATCCATACGGAATTCCAGATGACCACC-3k
METGFP1	5k-GGAATTCGCG GCCGCTCTAG AGTATGGATG GGGGTAATAG-3k
METGFP2	5k-GGAATTCGCG GCCGCTGGTG CTGGTATGAG TAAAGGAGAA GAACTTTTC-3k

marker cassette flanked by short homology regions to the end of the MCH3 locus. The 1.7 kb PCR product was transformed into strain CEN.PK113-13D, selecting for resistance to G418 (200 mg/l) on YEPD agar plates, and used to replace the stop codon of MCH3 by the 3′-HA–kanMX cassette. Thereby, the triple-HA epitope was fused in-frame to the carboxyterminal end of Mch3, allowing its detection with commercially available anti-HA antibodies. After transformation of this strain with plasmid pSH47, the kanMX marker was removed as described (Güldener et al., 1996).

Construction of MCH-GFP fusions

The multicopy vector p426MET25GFP with a URA3-selectable marker was constructed by PCR amplification of the plasmid p426MET25–GFP–ORF1 (kindly provided by B. Betz, Düsseldorf) using primers METGFP1 and METGFP2 (Table 1), digestion of the 6.3 kb DNA fragment with EcoRI and religation. The vector carries the methionine-repressible MET25 promoter, followed by an EcoRI restriction site, the GFP-coding sequence and the CYC1 termination region. The monocarboxylate transporter-homologous (MCH) genes were amplified from strain CEN.PK113-13D with forward GENEPAIRS primers (Research Genetics) and the reverse RGFPMCH primers listed in Table 1, using whole-cell PCR with the Expand High Fidelity PCR system (Roche) for 10 amplification cycles. PCR products were further amplified for 20 cycles with primers T1-ORF and again the reverse RGFPMCH primers. At its 3′ end, the T1-ORF primer is homologous to the common sequences added to the 5′ ends of the GENEPAIRS primers (italic in Table 1); at its 5′ end, it is homologous to the end of the MET25 promoter of the vector p426MET25GFP. At their 3′ ends, the RGFPMCH primers are homologous to the ends of the MCH genes omitting the stop codons; at their 5′ ends, they are homologous to the first part of the GFP sequence of the vector p426MET25GFP. The later was linearized with EcoRI and transformed into the strain CEN.PK113-13D, together with the PCR-amplified MCH genes, selecting for uracil prototrophy after homologous recombination in yeast. Plasmids were re-isolated as described (Boles and Zimmermann, 1993), amplified in E. coli strain DH5aF′, analysed by restriction enzyme mapping and re-transformed into the strain CEN.PK113-13D.

Determination of monocarboxylate concentrations and b-galactosidase activity

For the determination of pyruvate consumption rates, strains were grown at 30°C in SM medium with 2% ethanol, washed in SM medium without a carbon source and then inoculated to the same optical density (OD_{600}=1–2) in SM medium with pyruvate as the sole carbon source. Aliquots were taken every hour, centrifuged at 13 000×g for 5 min, and pyruvate concentrations in the supernatants were determined enzymatically in imidazol buffer (50 mM imidazol, 10 mM $MgCl_2$, 100 mM KCl, 0.1 mM EDTA, pH 7) by measuring the decrease of NADH absorption at OD_{340} after adding L-lactate dehydrogenase (Roche). For the determination of monocarboxylate secretion, cells were grown at 30°C in SM medium with 2% glucose, washed and inoculated to the same optical density (OD_{600}=0.5) in SM medium with 2% glucose at 30°C. Aliquots were taken at different time points and cells removed by centrifugation at 13 000×g for 5 min. Glucose, monocarboxylate concentrations and ethanol were measured enzymatically, using commercially available test combinations (Roche and R-Biopharm). To determine the cytosolic lactic acid concentration, the plasma membrane of the yeast cells were selectively permeabilized with 10 mg/ml nystatin for 15 min at 30°C(Larsson et al., 2000; Hecker, Universität Köln, personal communication). b-Galactosidase activity was measured as described previously (Miller, 1972). Crude extracts were prepared, using glass beads for breaking the cells according to published procedures (Ciriacy and Breitenbach, 1979). Protein was determined according to the microbiuret method (Zamenhoff, 1957), with bovine serum albumin as a standard.

Transport assays

Yeast cells were grown in YEP or SM medium, supplemented with 0.5% (vol/vol) acetic acid (pH 6.0), as carbon source. Exponentially growing cells were harvested by centrifugation, washed twice in ice-cold deionized water and resuspended in ice-cold deionized water to a final concentration of about 25–45 mg (dry weight)/ml. 10 ml yeast suspension were mixed in 10 ml conical tubes with 30 ml 0.1 M potassium phosphate buffer, pH 5.0. After 2 min of incubation at 25°C in a water bath, the reaction was started by the addition of 10 ml

aqueous solution of the labelled acid at the desired concentration and pH value, and stopped by dilution with 5 ml ice-cold water. The reaction mixtures were filtered immediately through Whatman GF/Cmembranes, the filters washed with 10 ml ice-cold water and transferred to the scintillation fluid (Opti-phase HiSafe II; LKB FSA Laboratory Supplies, Loughborough, UK). Radioactivity was measured in a Packard Tri-Carb 2200 CA liquid scintillation spectrophotometer with disintegrations/min correction. The effect of non-labelled substrates on the initial uptake velocities of labelled acid was assayed by simultaneously adding the labelled and non-labelled substrates. The following radioactive labelled substrates were utilized: D,L-[1-^{14}C]lactic acid, sodium salt (Amersham) and [U-^{14}C]acetic acid, sodium salt (Amersham). Non-specific ^{14}Cadsorption to the filters and to the cells was determined by adding labelled acid after ice-cold water. The values estimated represent less than 5% of the total incorporated radioactivity. The transport kinetics best fitting the experimental initial uptake rates and the kinetic parameters were determined by a computer-assisted non-linear regression analysis (GraphPAD Software, San Diego, CA, USA).

Isolation of mitochondria

Isolation of mitochondria was performed as described by Gawaz et al. (1990), based on a protocol by Daum et al. (1982). Briefly, the cells were harvested in exponential growth phase, incubated in Tris–Cl 0.1 M, pH 9.4, dithiothreitol (DTT) 10 mM, at 32°Cfor 10 min, washed and incubated in sorbitol 1.2 M, KP_i 20 mM, pH 7.4, containing 1 mg Zymolyase 20T (ICN Biochemicals)/g (wet weight) for 45 min. The protoplasts were resuspended in mannitol 0.6 M, Tris 10 mM, pH 7.4, bovine serum albumine (BSA) 0.1%, phenylmethylsulphonylfluoride (PMSF) 1 mM and homogenized with a Dounce homogenizer (12 strokes, tight-fitting pestle). After a low speed centrifugation (10 min, 1000×g) mitochondria were pelleted by centrifuging the supernatant at 9750×g for 10 min. The mitochondria were resuspended in mannitol 0.5 M, Tris 10 mM, pH 7.4, and stirred for 5 min. After centrifugation for 10 min at 1000×g, the final mitochondrial preparation was obtained by recentrifugation of the supernatant at 9750×g for 10 min.

Mitochondrial respiration

Mitochondrial respiration was measured at 30°C with a Clark oxygen electrode (Hansatech, Norfolk, UK) in a cell containing 2 ml sucrose 0.3 M, ethylene glycol-bis-(b-aminoethylene)-N,N,N′,N′-tetraacetic acid (EGTA) 1 mM, 3-(N-morpholino)-propanesulphonic acid (MOPS) 5 mM, KH_2PO_4 5 mM, BSA fatty acid-free 0.1%, pH 7.4. After addition of mitochondria to a final concentration of 150 mg protein/ml, malate (pH 7.4) was added to a final concentration of 0.5 mM. The rise in oxygen consumption rate after addition of varying concentrations of pyruvate (pH 7.4) was recorded using OXYGRAPH software (Hansatech). The obtained values were fitted to a hyperbolic curve using SigmaPlot software (SPSS, Erkrath, Germany) to obtain the kinetic parameters.

Pyruvate uptake into isolated mitochondria

For the transport assays, freshly isolated mitochondria were used, not later than 4 h after preparation. The mitochondria (0.5 mg protein) were incubated at 25°Cin sucrose 0.3 M, EGTA 1 mM, MOPS 5 mM, KH_2PO_4 5 mM, BSA fatty acid-free 0.1%, antimycin A 1 mg/ml, N,N,N′,N′-tetramethyl-p-phenylenediamine (TMPD) 1.5 mM, ascorbate 3 mM, [6,6′(n)-^{3}H]-sucrose 240 kBq, pH 7.4, 730 ml final volume for 2 min. The transport reaction was started by adding 585 ml of this mixture to 65 ml [2-^{14}C]-pyruvate (1 mM, 2 kBq/mmol). After 15, 30, 60, 120, 180 and 360 s, 100 ml aliquots were taken and pipetted into chilled reaction tubes containing 1 ml 1 M a-cyano-4-hydroxycinnamate to stop the transport reaction (Halestrap, 1975). After the last aliquot had been taken, 90 ml each aliquot were centrifuged through a silicone oil layer (AR 200, ρ=1.04 g/cm^3; Wacker Chemie, Germany) into 30 ml 0.5% SDS/15% sucrose to separate the mitochondria from the surrounding medium (Dawson et al., 1987). The radioactivity in the 0.5% SDS/15% sucrose was counted in Rotiszint Eco-Plus Scintillation Liquid (Roth, Germany) in a Beckman LS 6500 scintillation counter, using a double label program to measure ^{14}C-pyruvate and ^{3}H-sucrose simultaneously. The values obtained for pyruvate were corrected for the sucrose-permeable space (Dawson et al., 1987). SigmaPlot software (SPSS, Erkrath, Germany) was used to determine initial transport rates.

Results and discussion

Deletion of all monocarboxylate transporter-homologous genes

Five different genes (YDL054c, YKL222w, YNL125c, YOL119c and YOR306c) were identified in the yeast genome that encode proteins with similarities to the mammalian monocarboxylate transporters [André, 1995; Nelissen et al., 1997; Yeast Transport Protein database (YTPdb) (http://alize.ulb.ac.be/YTPdb/)]. Therefore, we called these genes MCH1–5, respectively, for monocarboxylate transporter homologues. The physiological functions and substrate specificities of these proteins were completely unknown. It has been reported previously that the growth of a yol119c yor306c double disruptant in acetate, lactate and pyruvate was similar to that of the parental strain (Lafuente and Gancedo, 1999).

In order to characterize the function of the highly redundant Mch family of S. cerevisiae, we deleted all five genes in the yeast strain CEN.PK113-13D by a succession of one-step gene deletions, using a loxP–kanMX–loxP resistance marker designed for repeated use (Güldener et al., 1996). Deletion of all genes from MCH1 to MCH5 yielded the mch1–5 mutant strain JMY18. The growth properties of the mch1–5 deletion strain were characterized in detail on SM medium agar plates with pyruvate, acetate, lactate, glucose or ethanol as the sole source of carbon, all in a concentration of either 2% or 0.1%. Under all conditions tested, the mch1–5 deletion strain did not exhibit any growth defects compared to the CEN.PK113-13D wild-type strain (data not shown). Similarly, the specific growth rates and the biomass yields of YEP batch cultures with glucose, glycerol, ethanol or lactate, the glucose consumption and ethanol production rates, and the ethanol consumption rates were nearly identical when compared between the mch1–5 mutant and the CEN.PK113-13D wild-type strain (data not shown).

In contrast, additional deletion of the JEN1 gene, which has been shown to be involved in the transport of lactate and pyruvate (Casal et al., 1999; Akita et al., 2000), led to a strongly reduced growth on SM medium with either lactate or pyruvate as the carbon source (Figure 1), but not with acetate, glucose or ethanol (not shown), all at a concentration of either 2% or 0.1%. In agreement with these observations, the consumption of

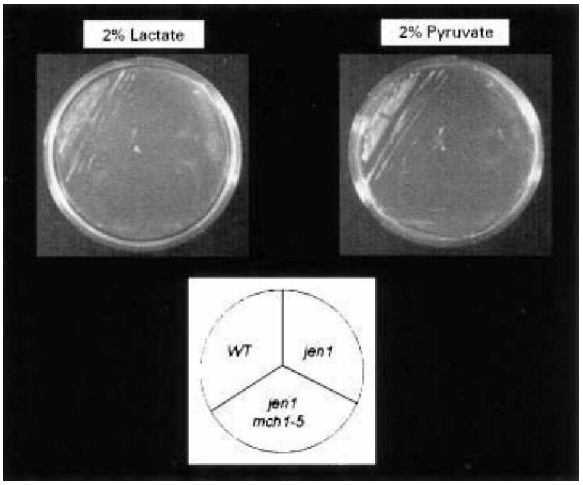

Figure 1. Growth of the jen1, jen1 mch1–5 and wild-type strains with monocarboxylates as the carbon source. The wild-type strain CEN.PK113-13D, the jen1 deletion strain and the jen1 mch1–5 deletion strain were streaked on solid SM media, supplemented with uracil and 2% pyruvate or 2% lactate. The plates were incubated at 30uC for up to 8 days

pyruvate was completely abolished in the strains deleted for the JEN1 gene, whereas the pyruvate consumption rate of the mch1–5 mutant cells was identical to that of wild-type cells (Figure 2).

The YHL008c gene of S. cerevisiae has been postulated to code for an acetate proton symporter (Paulsen et al., 1998) because it shows distinct similarities to the bacterial formate–nitrite transporters. This gene was additionally deleted in the jen1 mch1–5 mutant strain. The growth rate of the yhl008c jen1 mch1–5 sevenfold deletion mutant on SM medium with 2% or 0.1% acetate was identical to that of the corresponding wild-type strain (data not shown).

Uptake of monocarboxylate substrates

The mechanisms of transport of monocarboxylic acids in S. cerevisiae have already been described for the strains IGC4072 (Cássio et al., 1987; Casal et al., 1996) and W303 (Casal et al., 1995). It was also previously shown that the product of JEN1 is a permease for monocarboxylates, inducible by lactic acid (Casal et al., 1999). A mutant disrupted in this gene did not show measurable activity for lactate permease. In contrast, the transport of acetate was operational, confirming the model that at least two independent monocarboxylate permeases exist, distinct in their specificity and mechanisms of

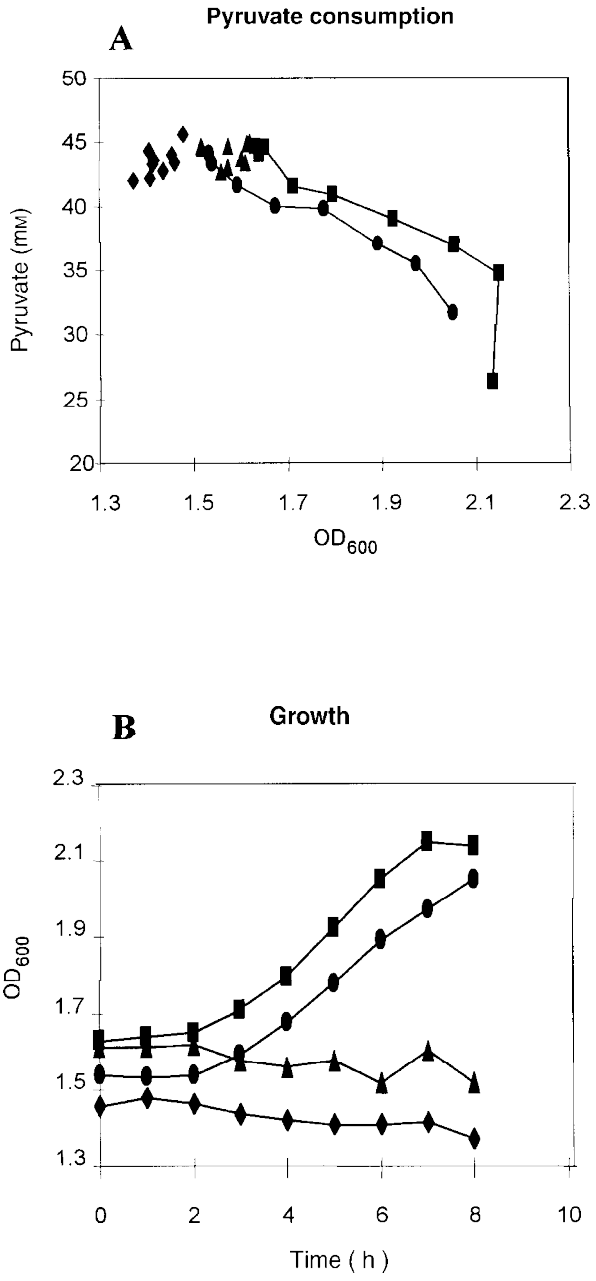

Figure 2. Pyruvate consumption of the wild-type strain CEN.PK113-13D ($), jen1 (+), jen1 mch1–5 (2) and mch1–5 (&) mutant strains. The cells were grown in SM medium supplemented with uracil and 2% ethanol, washed in the same SM medium without a carbon source, inoculated in SM medium with 0.4% pyruvate to OD_{600}=1.5 and incubated at 30uC. Aliquots were taken every hour for enzymatic determination of pyruvate concentration in the supernatant (A) and for measurements of the optical density (OD_{600}) (B)

regulation. Considering these results, in the present work we intended to clarify the role of the MCH genes, particularly regarding their involvement in acetate permease activity.

The strain CEN.PK, used in this work, has been extensively utilized in yeast research over the past years. This strain and other strains of this family were proposed to be used as reference strains for quantitative studies on S. cerevisiae due to their genetic and physiological properties (van Dijken et al., 2000). To determine whether there were any differences between the strains referred above, with respect to the transport of monocarboxylic acids, we performed a characterization of membrane transport properties of those substrates in the CEN.PK strain.

Initial uptake rates of labelled acetic acid and lactic acid at pH 5.0 were measured in cells grown in mineral medium containing acetic acid as the sole carbon and energy source. Under these growth conditions, the transport of lactic acid obeyed a simple diffusion mechanism, whereas a mediated transport system was found for the labelled acetic acid (Figure 3A). Studies regarding the specificity of this permease were carried out, showing that the system is competitively inhibited by cold propionic and acetic acids, indicating that these acids share the same transport system. Furthermore, the addition of cold lactic acid did not affect the transport of labelled acetic acid, reinforcing the fact that lactic acid is not transported by the system (not shown). The results obtained were in accordance with those previously known for other strains. Similar experiments were performed in cells grown in YEP medium containing acetic acid. Under this growth conditions, initial uptake rates of both labelled lactic acid and acetic acid (pH 5.0) followed a Michaelis–Menten kinetics, showing that a mediated transport system was found for the acids (Figure 3B). The uptake of both labelled acetic and lactic acid at pH 5.0 was also measured in YEP acetic acid-grown cells of the mch1–5 deletion mutant, the jen1 deletion mutant and the yhl008c jen1 mch1–5 sevenfold deletion mutant (Table 2). From the analysis of the data it is possible to conclude that only the disruption of the gene JEN1 abolished the uptake of lactate. None of the ORFs analysed accounts for the transport of acetate in S. cerevisiae. Therefore, the identity of their true substrates must await further experiments.

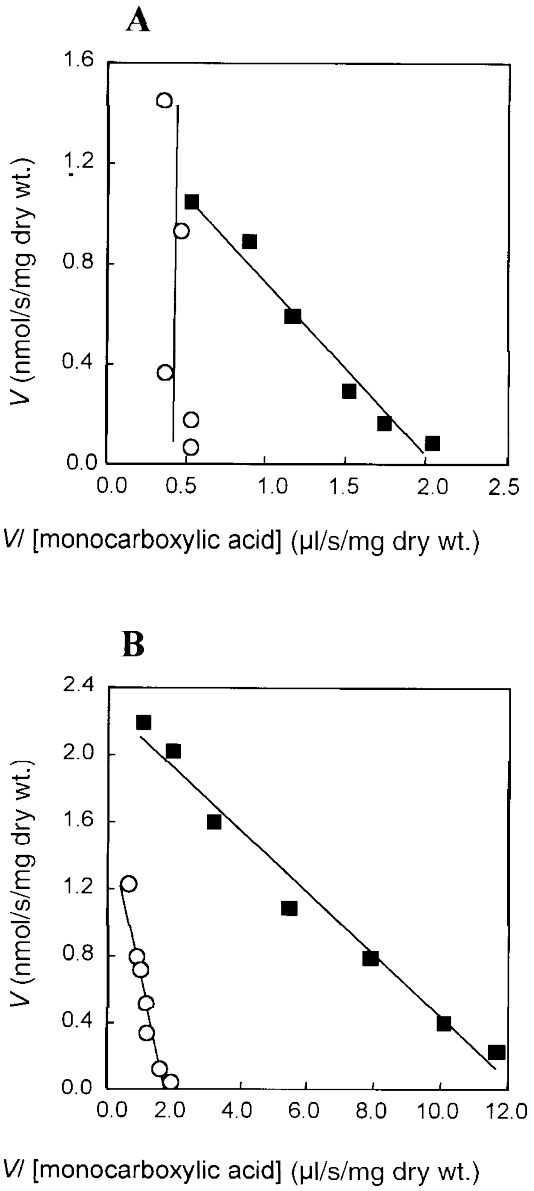

Figure 3. (A) Eadie–Hofstee plots of the initial uptake rates at pH 5.0 of labelled acetic acid (&) and lactic acid (##) of YNB acetic acid-grown CEN.PK 113-13D cells. (B) Eadie– Hofstee plots of the initial uptake rates at pH 5.0 of labelled acetic acid (&) and lactic acid (##) of YEP acetic acid-grown CEN.PK 113-13D cells

Secretion of monocarboxylates

During growth on glucose, yeast cells synthesize pyruvate, lactate and acetate and secrete these monocarboxylates into the growth medium. To test whether one of the MCH genes is involved in the secretion of monocarboxylates, extracellular concentrations of pyruvate, lactate and acetate were determined for the mch1–5 deletion mutant during growth on glucose. However, no differences could be observed as compared to the wild-type cells (Figure 4). Moreover, the intracellular concentrations of lactate in cytosolic or total cell extracts were also the same as in wild-type cells.

Transcriptional regulation of monocarboxylate transporter-homologous genes

In many cases, the expression of genes involved in the metabolism of specific substrates is regulated by those substrates. To analyse whether transcription of MCH genes is regulated by monocarboxylate substrates, the individual MCH coding regions were replaced by a lacZ–kanMX reporter cassette in the strain CEN.PK113-13D. Determination of b-galactosidase activities during growth of the yeast cells in media containing different carbon sources (2% lactate, glucose and ethanol) revealed that MCH5 was the highest-expressed MCH gene, followed by MCH4, MCH3 and MCH1, whereas transcription of MCH2 was hardly detectable. Transcription of MCH1 and MCH4 was repressed by lactate as compared to the other carbon sources, whereas transcription of MCH5 was slightly induced by lactate (Table 3). However, X-gal overlay assays with cells incubated in the presence of other carbon sources (2% pyruvate, acetate, succinate and 0.1% oleic acid) did not reveal any differences in comparison to the wild-type (data not shown). Thus, none of the MCH genes is repressed by glucose or is completely dependent on the presence of lactic acid, in contrast to the reported monocarboxylate transport systems of S. cerevisiae (Cássio et al., 1987; Casal et al., 1995, 1996).

Localization of monocarboxylate transporter-homologous proteins

To determine the intracellular localization of the Mch proteins, the MCH genes were fused in-frame to the green fluorescent protein (GFP) gene sequence on the multicopy vector p426MET25GFP, under the control of the methionine-repressible MET25 promoter. The plasmids were transformed into the wild-type strain CEN.PK113-13D, and cells were grown in SC medium with 2% glucose, but without uracil and methionine, into the exponential growth phase. Fluorescence microscopy revealed intracellular

Table 2. Kinetic parameters for the transport of monocarboxylic acids in *S. cerevisiae* strain CEN.PK113-13D and mutants disrupted in the genes indicated

Strain	Kinetic parameters			
	Acetic acid		Lactic acid	
	K_m (mM)	V_{max} (nmol/s/mg dry wt)	K_m (mM)	V_{max} (nmol/s/mg dry wt)
CEN.PK 113-13D	0.17±0.14	2.27±0.61	2.43±0.51	3.22±0.44
Dmch1–5	0.23±0.04	2.43±0.13	2.56±0.70	3.23±0.67
Djen1	ND	ND	NPA	NPA
Dyhl008c, mch1–5, jen1	0.24±0.04	2.11±0.11	NPA	NPA The cells were grown in YEP medium supplemented with acetic acid (0.5%, v/v) as carbon source. The uptake assays were done at pH 5.0. The parameters were estimated using the GraphPAD program.

ND, not determined. NPA, no permease activity.

localizations for all Mch–Gfp fusion proteins (Figure 5) which, however, could not be unequivocally attributed to certain structures in every case. Only Mch4–Gfp, a vacuolar membrane localization, could be predicted, as the protein was arranged in a large circular structure within the cells. Moreover, Mch3 seemed to be localized in the mitochondrial membrane, as could be revealed in comparison with DAPI staining (Figure 5B). To further study the subcellular localization of Mch3, we epitope-tagged the gene at its C-terminus with the HA-tag. After subcellular fractionation of Mch3–HA cells, we found the protein in the mitochondrial fraction (Figure 6, lane 5). The second washing step to enrich the mitochondria yielded an even stronger HA signal (Figure 6, lane 6), indicating a mitochondrial localization. Interestingly, the rat monocarboxylate transporter Mct1 has also recently been shown to reside in cardiac and skeletal muscle mitochondria (Brooks et al., 1999). To our knowledge, rat Mct1 and yeast Mch3 are the only members of the major facilitator superfamily that have been found to be localized in mitochondria.

Growth in aerobic glucose-limited chemostat cultures

To further study a potential role of the Mch proteins in carbohydrate metabolism, the biomass yields in aerobic glucose-limited chemostat cultures were determined. After at least 50 h, i.e. five residence times, the cultures reached a steady state. Samples were taken over a period of several hours and the dry weight of the samples was determined. Glucose was not detectable in these samples, confirming that the steady state was glucose-limited. The CEN.PK113-13D wild-type strain showed a biomass yield of 0.51±0.03 g/g glucose, precisely in agreement with data obtained by another group, who reported values of 0.52±0.01 g/g glucose (Pronk et al., 1994) and 0.50 g/g glucose (Postma et al., 1989) for other strains growing under the same growth conditions. The biomass yield of an mch3 deletion mutant, however, was significantly lower (Table 4). The reduction in biomass yield was even more pronounced in the mch1–5 mutant strain. This strain had a biomass yield of only 0.37±0.02 g/g glucose.

Pyruvate-dependent oxygen consumption and pyruvate transport into isolated mitochondria

The reduced biomass yields of the mch3 and mch1–5 mutant strains are reminiscent of a mutant impaired in mitochondrial pyruvate dehydrogenase activity (Pronk et al., 1994). In the case of the pyruvate dehydrogenase mutant, this was explained by an ATP-consuming bypass pathway providing acetyl co-enzyme A, comprising pyruvate decarboxylase, acetaldehyde dehydrogenase and acetyl co-enzyme A synthetase. As pyruvate is the substrate of mitochondrial pyruvate dehydrogenase, a mutant impaired in mitochondrial pyruvate uptake is expected to exhibit the same phenotype. Therefore, it was tempting to speculate that Mch3, together with another Mch protein, is involved in the uptake of pyruvate into mitochondria.

First, the kinetic parameters of pyruvate-dependent oxygen consumption of mitochondria

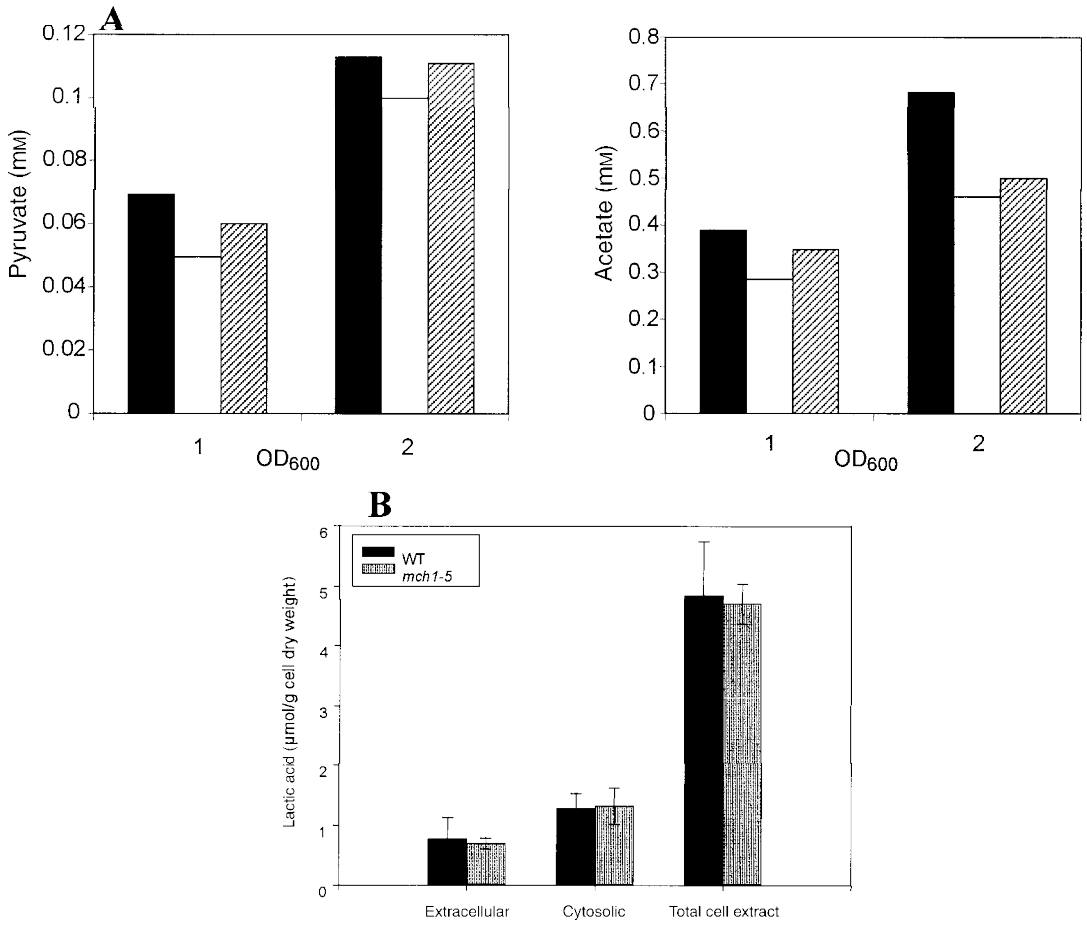

Figure 4. (A) Secretion of acetate and pyruvate in the wild-type strain CEN.PK113-13D (black bars), the mch1–5 (white bars) mutant and the jen1 mch1–5 (hatched bars) mutant strains. The cells were incubated at 30uC in SM medium supplemented with uracil and 2% glucose to an OD_{600}=0.5. Probes were taken at different optical densities for enzymatic determination of pyruvate and acetate concentrations in the supernatant. (B) L-Lactic acid in the wild-type strain CEN.PK113-13D and the mch1–5 mutant strain, grown on YEP medium with 2% glucose. A yeast cell suspension in PIPES buffer was shaken for 15 min at 30uC ('extracellular'), shaken for 15 min at 30uC with 10 mg/ml nystatin ('cytosolic') or shaken in a glass mill for 10 min at 4uC ('total cell extract'). The lactate concentration in the supernatants was determined enzymatically. Error bars depict the standard deviation of three independent measurements

isolated from the wild-type strain and the mch3 and mch1–5 deletion mutant strains were determined in the presence of 0.5 mM malate. Wild-type mitochondria showed a K_m of 0.28±0.03 mM, and mch3 and mch1–5 mitochondria both had a K_m of 0.4±0.1 mM. The oxygen consumption rates were similar in all three cases.

Second, the pyruvate uptake rates of mitochondria isolated from the wild-type and the mch1–5 mutant strain, grown on YEP-medium with 3% glycerol as the carbon source, were determined. The electron transport chain was inhibited by adding 1 mg/ml antimycin A. The mitochondria were energized by addition of TMPD/ascorbate as an electron donor for complex IV. Under these conditions, mitochondria showed cinnamate-inhibitable pyruvate uptake with characteristic transport kinetics (not shown). The initial transport rates differed considerably between individual preparations of mitochondria, and more importantly the transport activity of the isolated mitochondria declined over a period of a few hours during the measurements, resulting in very high standard deviations of the v_0 values obtained. With 0.1 mM pyruvate, wild-type mitochondria showed initial transport rates of 0.5±0.4 nmol/min/mg protein

Table 3. Specific b-galactosidase activities of MCH–promoter–lacZ fusion-containing yeast strains on different carbon sources

gene::lacZ-kanMX4	2% Lactate	2% Glucose	2% Ethanol
mch1	0.28	1.54	2.02
mch2	<0.1	0.12	0.2
mch3	2.79	2.05	2.35
mch4	2.61	5.47	6.05
mch5	24.97	17.46	7.18

The cells were grown overnight in SM media supplemented with uracil and various carbon sources (2% lactate, glucose or ethanol), transferred to the same fresh media and grown to $OD_{600\ nm}$=1–2. After harvesting the cells protein extracts were prepared and protein concentrations and b-galactosidase activities were measured. Data represent the average of two independent experiments.

(11 determinations), whereas in mch1–5 mitochondria a value of 0.7±0.4 nmol/min/mg protein (six determinations) was measured. Mitochondria of both strains preincubated with 10 mM a-cyano-4-hydroxycinnamate showed residual transport activity with a transport rate of 0.2±0.1 nmol/min/mg protein (five determinations). Therefore, if there is any involvement of the Mch proteins in mitochondrial pyruvate transport, this is only a minor involvement and not responsible for the strongly reduced biomass yield.

Conclusions

Our data give no evidence for an involvement of MCH1–5 and YHL008c in monocarboxylate transport in yeast. Of course, we cannot completely exclude that one or more of the proteins have minor activities which, however, are masked by the major monocarboxylate transport systems. The direct demonstration that the Mch proteins are not able to transport monocarboxylic acids requires their purification and reconstitution in liposomes. Nevertheless, this will hardly clarify their real functions. Therefore, the function of this highly redundant transporter family still remains unsolved, as does the nature of the actual acetate–propionate–formate transporter in yeast. On the other hand, our data strengthen the evidence that Jen1 indeed is a lactate transporter and not only a regulator of monocarboxylate permeases.

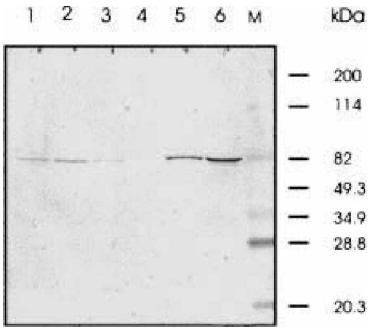

Figure 6. Localization of Mch3 in the mitochondrial fraction. Mitochondria from an Mch3-HA-expressing yeast strain were isolated and subjected to Western blot analysis with an anti-HA antibody (Anti-HA Y-11, Santa Cruz, USA) and AP-conjugated anti-rabbit IgG (Sigma). Each lane contains 10 mg protein. 1, protoplasts; 2, first low-speed pellet; 3, first low-speed supernatant; 4, first high-speed supernatant (cytoplasm); 5, first high-speed pellet (mitochondrial fraction); 6, second high-speed pellet (washed mitochondria)

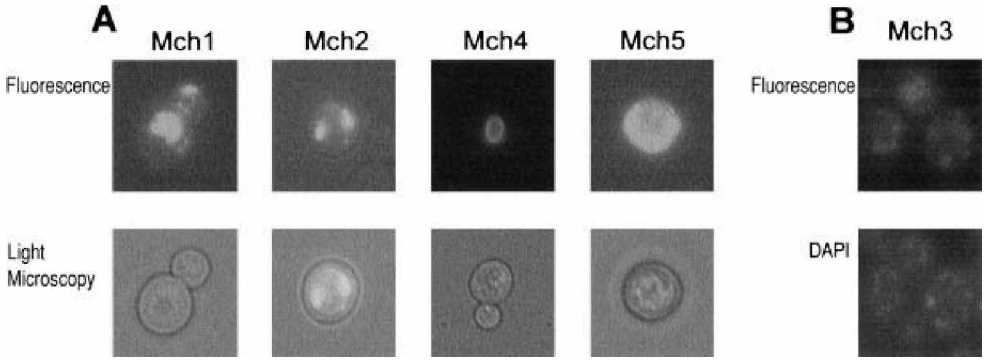

Figure 5. Fluorescence microscopy of yeast cells expressing Mch–Gfp fusion proteins during growth on glucose. The wild-type strain CEN.PK113-13D was transformed with plasmids expressing individual MCH–GFP fusion genes and incubated at 30uC for 3 h in SC medium without uracil and methionine but with 2% glucose. For DAPI staining (B), DAPI was added at 1 mg/ml to the cells 30 min before harvesting

Table 4. Growth yields of S. cerevisiae CEN.PK113-13D wild-type, the mch3 mutant and the mch1–5 mutant strain in aerobic glucose-limited chemostat cultures

Strain	Biomass yield (g (dry wt)/g glucose)
CEN.PK113-13D	0.51±0.03
Dmch3	0.47±0.02 ($p<0.005$)
Dmch1–5	0.37±0.02 ($p<0.005$)

Growth conditions: D=0.1/h, pH 5.0, T=30uC, S_R=5 g/l. Data are presented as means±SD of dry weight determinations of samples taken over at least 15 h of one steady-state culture each.

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