Differentially regulated malate synthase genes participate in carbon and nitrogen metabolism of *S.cerevisiae*

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ABSTRACT

We have isolated a second gene (*MLS1*), which in addition to *DAL7*, encodes malate synthase from *S.cerevisiae*. Expression of the two genes is specific for their physiological roles in carbon and nitrogen metabolism. Expression of *MLS1*, which participates in the utilization of non-fermentable carbon sources, is sensitive to carbon catabolite repression, but nearly insensitive to nitrogen catabolite repression. *DAL7*, which participates in catabolism of the nitrogenous compound allantoin, is insensitive to carbon catabolite repression, but highly sensitive to nitrogen catabolite repression. Results obtained with null mutations in these genes suggest that *S.cerevisiae* contains at least one and perhaps two additional malate synthase genes.

INTRODUCTION

Glyoxylate is produced as an intermediate in both carbon and nitrogen metabolism in *Saccharomyces cerevisiae* (1, 2). Acetyl-CoA, produced from catabolism of non-fermentable carbon sources such as fatty acids, ethanol or acetate, is condensed with glyoxylate to yield malate. This irreversible condensation reaction is catalysed by malate synthase and is the second specific step of the glyoxylate cycle (2). Malate produced in this fashion is used as a gluconeogenic precursor being converted to glucose or related metabolites via phosphoenolpyruvate (2). Malate also fulfills an anaplerotic function being converted to isocitrate; this replenishes the tricarboxylic acid cycle intermediates and thereby provides a source of glyoxylate via the isocitrate lyase reaction (3). Consistent with these physiological functions, production of malate synthase has been reported to be sensitive to carbon catabolite repression (4). When non-fermentable carbon sources are provided, the enzyme levels are at their maximum (4).

Glyoxylate is also a byproduct when allantoin is catabolised as a nitrogen source (1). It is produced by the reversible ureidoglycollate hydrolase-catalysed reaction (5). Since glyoxylate

is a chemically reactive compound, its accumulation in the cell would be highly toxic. The route through which glyoxylate, produced in allantoin catabolism, was eliminated from the cell was not investigated in early studies of allantoin metabolism. However, during isolation of the *DAL* gene cluster encoding all of the structural genes required for conversion of allantoin to urea, the *DAL7* gene was discovered (6). This gene was shown to be required for allantoin degradation, and its expression exhibits all regulatory characteristics of the other allantoin pathway genes; it is induced by the allantoin pathway inducer, allophanate or its gratuitous analogue, oxalurate (OXLU) and is sensitive to nitrogen catabolite repression (6). Recently, the *DAL7* protein sequence was recognized to be nearly identical to those of a number of plant and bacterial genes encoding malate synthase (7, 8).

Since yeast cells can grow in glucose-allantoin minimal medium, a condition that should result in strong carbon catabolite repression of malate synthase production, it was possible that this enzyme was encoded by more than one gene. Moreover, if malate synthase activity is encoded only by *DAL7* and therefore subjected to nitrogen catabolite repression, growth of yeast cells on media containing acetate or ethanol and nitrogen sources like ammonium sulfate would be impossible. This was an additional hint for another gene encoding malate synthase. To test this, we searched for a second gene encoding malate synthase and, after finding it, compared the regulation of its expression to that of *DAL7*. The two genes, though nearly identical in structure, are regulated quite differently in keeping with their physiological functions.

MATERIALS AND METHODS

All enzymes used were purchased from Boehringer-Mannheim or New England Biolabs Inc. and used as suggested by the supplier. Oligonucleotides were purchased from the Institute of Molecular Pathology, Vienna, Austria and the University of Tennessee Molecular Resource Center.

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Table 1. Strains used in this work

Strain	Genotype	Reference
HP49	MATa leu2 his3 ura3 trp1 pep4 cir ^o	This work
GA1-8C	MATa leu2 ura3-52 his3 trp1-1 ctt1 gal2	(11)
JR85	MATa leu2 ura3-52 his3 trp1 ctt1 gal2 mls1Δ::LEU2	This work
JD1	MATa leu2 ura3-52 his3 trp1 ctt1 gal2 dal7::HIS3	This work
JR86	MATa leu2 ura3-52 his3 trp1 ctt1 gal2 mls1Δ::LEU2 dal7::HIS3	This work
Σ1278b	MATα	(42)
TCY16	MATa lys5 ura3 dal80::hisG-URA3-hisG	(42)
W303-1a	MATa leu2-3 ura3-1his3-11,15 ade2-1 trp1-1 can1-100	(8)

Yeast strains and growth conditions

Yeast strains used throughout the study appear in Table 1. *E. coli* strain HB101, used in this work, has been described before (8). Yeast was transformed by the lithium acetate method (9), with plasmids or with linear fragments (10).

For experiments depicted in Figure 7, (Growth curves in SC-media) cells were precultured over night in 0.67% Yeast Nitrogen Base without amino acids, 2% glucose and an appropriate mixture of amino acids plus adenine and uracil (SC-medium). Cells were harvested by centrifugation (glucose was still present) and resuspended to a A_{600} of 0.02 in fresh media containing either 3% ethanol or 2% potassium acetate (pH 6.0) as sole carbon source instead of glucose. For experiments depicted in tables 2 and 3, yeast cells were grown in liquid medium containing 1% yeast extract, 2% peptone and either 2% ethanol, 2% potassium acetate (pH 6.0), or 0.2% oleate and resuspended to an A_{600} of 0.02. For growth on YPD containing 4% glucose, cells from the preculture were diluted 1:5000. After incubation at 30° with shaking for 20 hrs cells of all different cultures were harvested and RNA isolated as described earlier YS0020T(11). DNA-fragments were labelled using the random-primed labelling kit from Boehringer Mannheim. For experiments depicted in Figures 5, 6, 8, and 9, yeast cultures were grown in either Wickerham's minimal medium (12), or YNB (without amino acids and ammonia). Carbon sources were either 2% glucose or 2% sodium acetate (pH 6.0) and the nitrogen source as indicated (0.1%). All auxotrophic requirements were supplemented as necessary. Cultures were grown at 30°C to mid-log phase (40–60 Klett units). Total RNA was isolated from these cultures by the method of Carlson and Botstein (13) and passed twice over oligo-dT cellulose columns (Pharmacia). Poly(A)⁺ RNA was resolved and transferred to Gene Screen Plus[™] Nylon 66 or Schleicher and Schuell Nytran^R nylon membranes.

Isolation and sequencing of *MLS1*

Parts of the *MLS1* gene from *S. cerevisiae* were cloned by PCR using degenerate primers encoding regions highly conserved in the family of malate synthases: 5'-CCAAGC-TTGCATGCGTNGAPATHACNGGNCCNGTNG-APMGNAAPATG-3' where P = A or G, H = A, C, or T,

and M = C or A and 5'-CCAAGCTTGCATGCNGCNGCC ATNCCNCCCATNGC-3'. The template was genomic DNA from *S. cerevisiae* strain HP49, and the first five cycles were done with Klenow polymerase as originally described (14). The resulting mixture was precipitated, and aliquots were used as templates for 30 cycles of PCR with Taq polymerase (15). After phenol extraction and precipitation, the PCR-products were kinased, blunt ended with T4 polymerase, ligated, and digested with *Hind*III (the cloning site in the primers). The resulting fragments were separated by agarose gel electrophoresis, isolated, and cloned into pUC19.

Isolation of genomic DNA from yeast cells was performed by the methods of either Olson et al. (16) or Winston et al. (17). *E. coli* colony-hybridization, Southern-blot and Northern-blot procedures were as described (18–20). Preparation of phage and isolation of phage DNA has been described by Price et al. (21).

Overlapping sequence of a 3.5 kb *Hind*III DNA fragment containing the *MLS1* gene was determined using a set of ordered deletions generated by exonuclease III shortening (Promega Erase-a-base kit) of the parent fragment. The same methods were used for determination of overlapping sequences of an adjacent 0.7 kb *Hind*III fragment and an overlapping 3.5 kb *Bam*HI-*Sac*I fragment, both containing portions of the vector (Figure 1C). Commercially available universal and reverse primers were used throughout except in two instances where custom primers, 5'-CCATTGGGTGACAAGAAC-3' (hybridizing to *MLS1* sequence +1516 to +1533) and 5'-TACTACTTTGTTTAGTT-C-3' (hybridizing to the sequence from -25 to -43) were used instead.

Construction of *MLS1* and *DAL7* null mutants

The genomic copy of the *MLS1* gene was disrupted by transformation with a linear fragment consisting of the 5'-region between *Pst*I and *Sal*I (1.2 kb), a 0.7 kb *Bam*HI-*Sal*I fragment from the 3'-region of the *MLS1* gene and a *Sal*I-fragment containing the *LEU2* gene (2.0 kb) in place of the open reading frame of the *MLS1* gene (Figure 1A). The *LEU2* gene was inserted twice into the plasmid, however, and therefore the linear fragment used for disruption was 5.9 kb long instead of 3.9 kb. The corresponding plasmid was constructed piece by piece in

Figure 1. A. Restriction map of the plasmid (pAH785) used for deletion of the *MLS1* coding region. B. Restriction maps of the important regions of the plasmids (pHY39 and pHY40) used to generate the *dal7* disruption mutants. C. Restriction map of the genomic, *MLS1*-containing insert (6.8 kb yeast *EcoRI* fragment) from lambda clone 6517. Location of the sequenced region, the PCR-fragment and the ORF coding for malate synthase are indicated.

the order mentioned above in the cloning vector pUC18, since the straightforward approach by elimination of the *SalI*-fragment containing the ORF was unsuccessful. Construction of the plasmids used for disruption of the *DAL7* gene are shown in Figure 1B. The 7.4 kb *SacI* fragment was isolated from plasmids pHY39 and/or pHY40 and used to transform strains W303-1a or GA1-8C, respectively. His⁺ transformants were isolated. Southern blot genomic analysis was used to verify that the desired genomic disruption had been obtained (data not shown).

Malate synthase assays

Cells used for assay of malate synthase activity were harvested by centrifugation after 24 hrs of growth in the appropriate media,

washed twice with ice-cold distilled water, resuspended in 50mM phosphate buffer (pH 7.0) and broken with a Braun homogenizer (Braun-Melsungen AG, Germany). The enzyme assays were performed as described (22) with Elman's reagent determining the amount of coenzyme A released after five minutes ($E = 13,600 \text{ cm}^2/\text{M}$), omitting either acetyl CoA or glyoxylate in the assay mixtures of reference samples. Activity values are reported in Table 3 as micromoles per min. per mg protein. Urediglycollate hydrolase was assayed as described earlier (23).

Hybridization procedures

PolyA⁺ RNA was isolated and Northern blots were prepared as described earlier (20). The Northern blots were prehybridized

at 42°C in 50% deionized formamide, containing 1% sodium dodecyl sulfate and 1M NaCl for one to two hours. Hybridization was carried out for 12 to 16 hours under the same conditions except that the hybridization solution (which varied from 5 to 8 ml) also contained 300 micrograms of highly sheared calf thymus DNA. The total amount of probe per reaction mixture was 35 ng/ml of solution at a specific activity of 1.8×10^6 CPM per ml. Following hybridization, the filters were washed twice (10 to 15 min. each) at room temperature with $2 \times$ SSC (0.3M NaCl and 0.03M sodium citrate), twice (30 min. each) at 60°C with $2 \times$ SSC containing 1% sodium dodecyl sulfate, and twice (30 min. each) at room temperature with $0.1 \times$ SSC (0.015M NaCl and 0.0015M sodium citrate). The filters were then dried and autoradiographs prepared using standard procedures.

RESULTS

Isolation and nucleotide sequence determination of the MLS1 gene

The *S. cerevisiae* malate synthase (MLS1) gene was cloned with PCR-based procedures using degenerate primers hybridizing to highly conserved regions in the family of malate synthases (24–26). The regions used encoded Val₉₂ to Met₁₀₂ and Ala₃₂₆ to Ala₃₃₂, respectively, in the *E. coli* protein. Four PCR products (1200, 800, 600 and 300bp), were obtained and cloned into plasmid pUC19. Restriction mapping of the resulting plasmids identified six different inserts, which were sequenced from both ends. Only one of the inserts contained an open reading frame whose product showed homology to malate synthases. The insert

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                                     GTCGACGG
-460*          -440*          -420*          -400*
ATAGAAGCGGTTGTCCTTCCCGGCGAGCCGAGTCGGGCGGAGGTCGGATAAATTTGTATTGTGTTTGTATTCT
-380*          -360*          -340*          -320*
GTCATGAGTATTACTTATGTTCTCTTAGGTAACCCAGGTTAATCAATCACAGTTTCATACCGGCTAGTATTCAAATT
-300*          -280*          -260*          -240*
ATGACTTTTCTTCTGCACTGTGAGCCTTACGACGATTATCTATGAGCTTTGAATATAGTTTGCCGTGATTCTATCTTT
-220*          -200*          -180*          -160*
AATTGGATAAATAAATGCGAAGGATCGATGACCTTATTATTATTTTCTACACTGGCTACCGATTAACTCATCTTCT
-140*          -120*          -100*          -80*
TGAAAGTATATAAGTAACAGTAAATATACCGTACTTCTGCTAATGTTATTGTCCCTTATTTCTTTCTTCTCTTA
-60*          -40*          -20*
TGCTATAGTACCTAAGAATAACGACTATTGTTTGAACATAAACAAAGTAGTAAAGCACATAAAGAATTAAGAAAATG
Met
20*          40*          60*
GTT AAG GTC AGT TTG GAT AAC GTC AAA TTA CTG GTG GAT GTT GAT AAG GAG CCT TTC TTT
Val Lys Val Ser Leu Asp Asn Val Lys Leu Leu Val Asp Val Asp Lys Glu Pro Phe Phe
80*          100*          120*
AAA CCA TCT AGT ACT ACA GTG GGA GAT ATT CTT ACC AAG GAT GCT CTA GAG TTC ATT GTT
Lys Pro Ser Ser Thr Thr Val Gly Asp Ile Leu Thr Lys Asp Ala Leu Glu Phe Ile Val
140*          160*          180*
CTT TTA CAC AGA ACT TTC AAC AAC AAG AGA AAA CAA TTA TTG GAA AAC AGA CAA GTT GTT
Leu Leu His Arg Thr Phe Asn Asn Lys Arg Lys Gln Leu Leu Glu Asn Arg Gln Val Val
200*          220*          240*
CAG AAG AAA TTA GAC TCG GGC TCC TAT CAT CTG GAT TTC CTG CCT GAA ACT GCA AAT ATT
Gln Lys Lys Leu Asp Ser Gly Ser Tyr His Leu Asp Phe Leu Pro Glu Thr Ala Asn Ile
260*          280*          300*
AGA AAT GAT CCC ACT TGG CAA GGT CCA ATT TTG GCA CCG GGG TTA ATT AAT AGG TCA ACG
Arg Asn Asp Pro Thr Trp Gln Gly Pro Ile Leu Ala Pro Gly Leu Ile Asn Arg Ser Thr
320*          340*          360*
GAA ATC ACA GGG CCT CCA TTG AGA AAT ATG CTG ATC AAC GCT TTG AAT GCT CCT GTG AAC
Glu Ile Thr Gly Pro Pro Leu Arg Asn Met Leu Ile Asn Ala Leu Asn Ala Pro Val Asn
380*          400*          420*
ACC TAT ATG ACT GAT TTT GAA GAT TCA GCT TCA CCT ACT TGG AAC AAC ATG GTT TAC GGT
Thr Tyr Met Thr Asp Phe Glu Asp Ser Ala Ser Pro Thr Trp Asn Asn Met Val Tyr Gly
440*          460*          480*
CAA GTT AAT CTC TAC GAC GCG ATC AGA AAT CAA ATC GAT TTT GAC ACA CCA AGA AAA TCG
Gln Val Asn Leu Tyr Asp Ala Ile Arg Asn Gln Ile Asp Phe Asp Thr Pro Arg Lys Ser
500*          520*          540*
TAC AAA TTG AAT GGA AAT GTG GCC AAC TTG CCC ACT ATT ATC GTG AGA CCC CGT GGT TGG
Tyr Lys Leu Asn Gly Asn Val Ala Asn Leu Pro Thr Ile Ile Val Arg Pro Arg Gly Trp
560*          580*          600*
CAC ATG GTG GAA AAG CAC CTT TAT GTA GAT GAT GAA CCA ATC AGC GCT TCC ATC TTT GAT
His Met Val Glu Lys His Leu Tyr Val Asp Asp Glu Pro Ile Ser Ala Ser Ile Phe Asp
620*          640*          660*
TTT GGT TTA TAT TTC TAC CAT AAT GCC AAA GAA TTA ATC AAA TTG GGC AAA GGT CCT TAC
Phe Gly Leu Tyr Phe Tyr His Asn Ala Lys Glu Leu Ile Lys Leu Gly Lys Gly Pro Tyr

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680*          700*          720*
TTC TAT TTG CCA AAG ATG GAG CAC CAC TTG GAA GCT AAA CTA TGG AAC GAC GTC TTC TGT
Phe Tyr Leu Pro Lys Met Glu His His Leu Glu Ala Lys Leu Trp Asn Asp Val Phe Cys

740*          760*          780*
GTA GCT CAA GAT TAC ATT GGG ATC CCA AGG GGT ACA ATC AGA GCT ACT GTG TTG ATT GAA
Val Ala Gln Asp Tyr Ile Gly Ile Pro Arg Gly Thr Ile Arg Ala Thr Val Leu Ile Glu

800*          820*          840*
ACT TTG CCT GCT GCT TTC CAA ATG GAA GAG ATC ATC TAT CAA TTA AGA CAA CAT TCT AGT
Thr Leu Pro Ala Ala Phe Gln Met Glu Glu Ile Ile Tyr Gln Leu Arg Gln His Ser Ser

860*          880*          900*
GGG TTG AAT TGC GGA CGT TGG GAC TAT ATT TTC TCT ACA ATC AAG AGA TTA AGA AAT GAT
Gly Leu Asn Cys Gly Arg Trp Asp Tyr Ile Phe Ser Thr Ile Lys Arg Leu Arg Asn Asp

920*          940*          960*
CCT AAT CAC ATT TTG CCC AAT AGA AAT CAA GTG ACT ATG ACT TCC CCA TTC ATG GAT GCA
Pro Asn His Ile Leu Pro Asn Arg Asn Gln Val Thr Met Thr Ser Pro Phe Met Asp Ala

980*          1000*          1020*
TAC GTG AAA AGA TTA ATC AAT ACC TGT CAT CGG AGG GGT GTT CAT GCC ATG GGT GGT ATG
Tyr Val Lys Arg Leu Ile Asn Thr Cys His Arg Arg Gly Val His Ala Met Gly Gly Met

1040*          1060*          1080*
GCT GCG CAA ATC CCT ATC AAA GAC GAC CCG GCA GCC AAT GAA AAG GCC ATG ACT AAA GTC
Ala Ala Gln Ile Pro Ile Lys Asp Asp Pro Ala Ala Asn Glu Lys Ala Met Thr Lys Val

1100*          1120*          1140*
CGT AAT GAT AAG ATT AGA GAG CTG ACA AAT GGA CAT GAT GGG TCA TGG GTT GCA CAC CCA
Arg Asn Asp Lys Ile Arg Glu Leu Thr Asn Gly His Asp Gly Ser Trp Val Ala His Pro

1160*          1180*          1200*
GCA CTG GCC CCT ATT TGT AAT GAA GTT TTC ATT AAT ATG GGA ACA CCA AAC CAA ATC TAT
Ala Leu Ala Pro Ile Cys Asn Glu Val Phe Ile Asn Met Gly Thr Pro Asn Gln Ile Tyr

1220*          1240*          1260*
TTC ATT CCT GAA AAC GTT GTA ACG GCT GCT AAT CTG CTG GAA ACC AAA ATT CCA AAT GGT
Phe Ile Pro Glu Asn Val Val Thr Ala Ala Asn Leu Leu Glu Thr Lys Ile Pro Asn Gly

1280*          1300*          1320*
GAG ATT ACT ACC GAG GGA ATT GTA CAA AAC TTG GAT ATC GGG TTG CAG TAC ATG GAA GCT
Glu Ile Thr Thr Glu Gly Ile Val Gln Asn Leu Asp Ile Gly Leu Gln Tyr Met Glu Ala

1340*          1360*          1380*
TGG CTC AGA GGC TCT GGA TGT GTG CCC ATC AAC AAC TTG ATG GAA GAC GCC GCC ACT GCT
Trp Leu Arg Gly Ser Gly Cys Val Pro Ile Asn Asn Leu Met Glu Asp Ala Ala Thr Ala

1400*          1420*          1440*
GAA GTG TCT CGT TGT CAA TTG TAT CAA TGG GTG AAA CAC GGT GTT ACT CTA AAG GAC ACG
Glu Val Ser Arg Cys Gln Leu Tyr Gln Trp Val Lys His Gly Val Thr Leu Lys Asp Thr

1460*          1480*          1500*
GGA GAA AAG GTC ACC CCA GAA TTA ACC GAA AAG ATT CTA AAA GAA CAA GTG GAA AGA CTG
Gly Glu Lys Val Thr Pro Glu Leu Thr Glu Lys Ile Leu Lys Glu Gln Val Glu Arg Leu

1520*          1540*          1560*
TCT AAG GCA AGT CCA TTG GGT GAC AAG AAC AAA TTC GCG CTG GCC GCT AAG TAT TTC TTG
Ser Lys Ala Ser Pro Leu Gly Asp Lys Asn Lys Phe Ala Leu Ala Ala Lys Tyr Phe Leu

1580*          1600*          1620*
CCA GAA ATC AGA GGC GAG AAA TTC AGT GAA TTT TTG ACT ACA TTG TTG TAC GAC GAA ATT
Pro Glu Ile Arg Gly Glu Lys Phe Ser Glu Phe Leu Thr Thr Leu Leu Tyr Asp Glu Ile

1640*          1660*          1680*
GTG TCC ACT AAG GCG ACG CCC ACT GAT TTG AGC AAA TTGTGATCTCCCTTGCCCCAGTGTACACATA
Val Ser Thr Lys Ala Thr Pro Thr Asp Leu Ser Lys Leu

1700*          1720*          1740*          1760*
TATAAAATATATTCATGCCTATATGATTATCGGAATCCAAAACTTATCTTTATATGCTTCGTTTCGTAGTTA
1780*          1800*          1820*
GCAATGAATCATCTTATCATGATTCCTTTTTTTGTTTTGGCCAGATTAATGTATCACGGTCGAC

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Figure 2. DNA sequence of the *MLS_I* gene, and the deduced amino acid sequence. The DNA sequence has been submitted to the EMBL data bank and given the accession number X64407 SCMLSIG. Features underlined or bracketed are described in the text.

(approx. 800 bp, designated PCR in Figure 1C) of this plasmid (pMLS9) was used to screen a YEp13 genomic library (27) for identification of a clone containing the gene. Three different

plasmids were isolated in this way, and their restriction maps determined (Figure 1C). Southern-hybridization with the DNA fragment used to screen the library indicated the location of the

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MLS1  MVKVSIDNVKLLVDVDEKPFFFPKSSSTTVGDILTTKDALEFIVLLERTFMNK 50
      | | | | | | | | | | | | | | | | | | | | | | | | | | | |
DAL7  MVKISIDNTALYADIDTTQQFEPSKTTVADILTKDALEFIVLLERTFMNST
      | | | | | | | | | | | | | | | | | | | | | | | | | | | |
      RKQLLENRQVVQKKLDSGSYHLDFLPETANIRNDPTWQGPILAPGLINRS 100
      | | | | | | | | | | | | | | | | | | | | | | | | | | | |
      RKQLLANRSLQSKLDSGEYRFDFLPETEQIRNDPTWQGAIPAPGLINRS
      | | | | | | | | | | | | | | | | | | | | | | | | | | | |
      TEITGPPILRNMLINALNAPVNTYMTDFEDSASPTWNMMVYGQVNLDAIR 150
      | | | | | | | | | | | | | | | | | | | | | | | | | | | |
      SEITGPPILRNMLVNDLNAEVTTYMTDFEDSSPTWNNIYGQVNLDAIR
      | | | | | | | | | | | | | | | | | | | | | | | | | | | |
      NQIDFDTPRKSYKLINGVANLPTIIVRPRGWMVEKHYVDDEPISASIF 200
      | | | | | | | | | | | | | | | | | | | | | | | | | | | |
      NQIDFKTPRKEYLKGDISRLPTLIVRPRGWMVEKHYIDDEPISASIF
      | | | | | | | | | | | | | | | | | | | | | | | | | | | |
      DFGLYFYHNAKELIKLGKGPYFYLPKMEHELEAKLMNDVFCVAQDYIGIP 250
      | | | | | | | | | | | | | | | | | | | | | | | | | | | |
      DFGLYFYHNAKELVKGKGPYFYLPKMEHEMEVKLMNDIFCVAQDYIGMP
      | | | | | | | | | | | | | | | | | | | | | | | | | | | |
      RGTIRATVLIETLPAAFQMEKIIYQLRQESSGLNCGRNDYIFSTIKRLRN 300
      | | | | | | | | | | | | | | | | | | | | | | | | | | | |
      RGTIRATVLIETLPAAFQMEKIIYQIRESSGLNCGRNDYIFSTIKRLRN
      | | | | | | | | | | | | | | | | | | | | | | | | | | | |
      DPNHILPNRNQVMTSPFMDAYVKRLINTCHRRGVHANGMAAQIPIKDD 350
      | | | | | | | | | | | | | | | | | | | | | | | | | | | |
      LNEHVLPNRDLVMTSPFMDAYVKRLINTCHRRGVHANGMAAQIPIKDD
      | | | | | | | | | | | | | | | | | | | | | | | | | | | |
      PAANEKAMTKVRNDKIRELTNGEDGSWAHPALAPICNEVFIMGTNPQI 400
      | | | | | | | | | | | | | | | | | | | | | | | | | | | |
      PKANEAMNKVRNDKIREMTNGEDGSWAHPALAPICNEVFSNMGTAHQI
      | | | | | | | | | | | | | | | | | | | | | | | | | | | |
      YFIPENVVTAANLLETQIPNGEITTEGIVQNLDIGLQYMEANLWGGSCVP 450
      | | | | | | | | | | | | | | | | | | | | | | | | | | | |
      YFVPDVEVTSSDLNLTQIQDAQVTEGIRVNLDIGLQYMEANLWGGSCVP
      | | | | | | | | | | | | | | | | | | | | | | | | | | | |
      INNLMEDAATAEVSRCQLYQWVKGVTLLDGTGKVTPELTKILKEQVER 500
      | | | | | | | | | | | | | | | | | | | | | | | | | | | |
      INNLMEDAATAEVSRCQLYQWVKGVTLLDGTGKVTPELTAKILNEETAK
      | | | | | | | | | | | | | | | | | | | | | | | | | | | |
      LSKASPLGKNNKFFALAAYFLPEIRGEKFSEFLTLLYDEIVSTKATPTD 550
      | | | | | | | | | | | | | | | | | | | | | | | | | | | |
      LASASPLGKNNKFFALAAYFLPEVTGKIFSDFLTLLYDEIIKPSAKPVD
      | | | | | | | | | | | | | | | | | | | | | | | | | | | |
      LSKL 554
      | | | |
      LSKL

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Figure 3. Comparison of the amino acid sequences deduced for the *MLS1* and *DAL7* genes. A bar between the sequences indicates identity, whereas double or single dots indicate greater or lesser similarities, respectively.

ORF of the putative malate synthase gene. Finally, three small fragments of the YEp13-inserts were isolated and cloned into Bluescript KS⁻ for sequencing: the 3.5 kb *Hind*III-fragment in both orientations, the 0.7 kb *Hind*III fragment containing parts of the vector (see restriction map), and the overlapping 3.5 kb *Sac*I-*Bam*HI fragment, containing approx. 2.5 kb of vector sequences.

Using standard dideoxy sequencing strategies, a sequence of the ORF was obtained (Figure 2). Both strands of the DNA fragments were completely sequenced. This sequence contained a 1,662 bp ORF devoid of introns. The junction between the ORF-containing insert and the YEP13 vector was extremely close to the 3'-end of the ORF. The stop codon, TGA, contained two bases of the *Sau*3A (GATC) recognition site of the junction to the vector YEp13. Therefore, to obtain a clone extending into the 3'-region of the gene, the original cloned PCR-fragment (0.8 kb) was used for hybridization to an ordered yeast genomic library of 855 lambda phage encompassing approximately 90% of the yeast genome (28). Two phages (6741 and 6517) containing inserts with overlapping restriction maps yielded positive signals. These phages contained inserts which were mapped to the third *Sfi*I fragment on the left arm of chromosome XIV, approximately 55 kb distal of *TOP2* (29, unpublished mapping data). From the

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S. cerevisiae  MLS1  5'-A T A A A A T G C G A A G-3'
               DAL7  5'-G A A A G T T G C G G T G-3'
               5'-G A A A A T T G C G T T T-3'
               DAL2  5'-C A A A C T T G C G C T T-3'
               DAL4  5'-G G A A A A T G C G C C C-3'
               5'-C A A A A T A G C G C C C-3'
               DUR1,2 5'-G A A A T G T G C G T T T-3'
               DUR3,4 5'-G A A A A A T G C G T A T-3'
               CAR2   5'-A A A A A A A G C G C G T-3'
               CAR2   5'-C A A A T G T G C G C A C-3'
H. polymorpha MAS  5'-G A A A A A T G C G G G T-3'
N. crassa acu-9    5'-G A A A A G T G C C G A G-3'

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Figure 4. Comparison of sequences situated 5' to the *MLS1* gene with upstream induction sequences (*UIS*) contained in the upstream regions of *S. cerevisiae* genes that respond to the allantoin pathway inducer, allophanate (8, 44, 45; Genbauffe and Cooper, unpublished observations; El Berry and Cooper, unpublished observations; 10). We also noticed that *UIS* elements are also situated upstream of the malate synthase genes of *H. polymorpha* and *N. crassa* (31, 33).

restriction map of the insert contained in phage 6517, we concluded that it contained the entire *MLS1* gene together with its 3' flanking region. These sequences confirmed that the 3' end of the ORF of the originally cloned DNA fragment ended in a stop codon that overlapped the *Sau*3A site at the junction between the YEp13 vector and its insert. The complete sequence of the ORF including its 5' and 3' flanking regions are shown in Figure 2.

Translation of the ORF depicted in Figure 2, predicted a 554 amino acid protein with a calculated molecular weight of 62,791 and a pI of 7.18 (30). Comparison of the predicted amino acid sequence of the ORF with those in the data base demonstrated 45 to 81% identity and 64 to 89% similarity with malate synthases isolated from *E. coli* to higher plants (24–26, 31–33). This result led us to conclude that there was a high probability that the ORF encoded a malate synthase gene which we designated *MLS1*. *MLS1* was 81% identical and 89% similar to the *S. cerevisiae* *DAL7* sequence (Figure 3), a gene previously reported to encode malate synthase (7, 8). The high homology between *MLS1* and *DAL7* extended to the DNA sequences which were 74% identical (data not shown). In many cases only the last base of a triplet was different. The *MLS1* protein sequence contains a putative peroxisomal targeting sequence at the C-terminus (SKL) as does the *DAL7* gene product (34, 35).

The 5' sequence contained a putative TATA motif at position –145 (underlined in Figure 2). In addition, at position –325, a consensus sequence found in promoters of genes encoding B-oxidation enzymes (36) was found (boldly underlined in Figure 2). This sequence might be involved in the regulation of the *MLS1* gene, although no experiments have yet been performed. It is not, however, observed upstream of *DAL7* (8). Found upstream of *MLS1* were five sequences (bracketed, in Figure 2) homologous to the *UAS_{NTR}*, the cis-acting element responsible for NCR-sensitive transcriptional activation of the *DAL* genes including *DAL7* (8, 37–39). In addition, a sequence (doubly underlined in Figure 2) homologous to the *UIS* element, mediating response of the *DAL7* gene to the allantoin pathway inducer, was also found (Figure 4) (8, 40).

Carbon and nitrogen responsive control of *MLS1* gene expression

Putative regulatory sequences upstream of *MLS1* and *DAL7* mentioned above suggested that control of the two genes'

Figure 5. Expression of the *DAL7* and *MLS1* genes in wild-type strain GA1-8C. Cultures were grown in either Wickerham's minimal medium with 2% glucose as carbon source or YNB medium with 2% acetate as carbon source. Nitrogen source was either 0.1% proline (PRO) or 0.1% asparagine (ASN). Where OXLU was used (PRO+), it was provided at a final concentration of 0.25 mM. OXLU was not added to cultures grown on 2% acetate because the pH was too high for OXLU transport (pH 3.3 optimum) to occur. 7 μ g of poly(A)⁺ RNA were loaded per lane. Probes used were random-primed-labelled plasmid pHY3 (*DAL7*), pMLS9(*MLS1*), and pTCM 3.2 (*TCM1*).

Figure 6. Expression of *MLS1* gene in wild-type Σ 1278b and *dal80* disruption mutant (TCY16) strains. Cultures were grown in Wickerham's minimal medium with 0.1% proline (PRO) as nitrogen source and 2% glucose as carbon source. Where OXLU was used (PRO+), it was provided at a final concentration of 0.25 mM. 7 μ g of poly(A)⁺ RNA were loaded per lane. Probes were random-primed labeled plasmid DNAs: pMLS9 (*MLS1*) and pTCM 3.2 (*TCM1*).

expression might share some characteristics in common, but also exhibit marked differences as well. To assess control of *MLS1* and *DAL7* expression, we prepared poly(A)⁺ RNA preparations from wild type cells grown in conditions of high and low carbon or nitrogen catabolite repression (Figure 5). When cultures were grown in glucose proline medium, a condition of high carbon catabolite repression but low nitrogen catabolite repression, *DAL7* mRNA was produced, but *MLS1* mRNA was not detected (Figure 5, lanes A and H). *DAL7* mRNA production was modestly responsive to the allantoin pathway gratuitous inducer, OXLU (PRO+) and was completely sensitive to nitrogen catabolite repression (Figure 5, lanes A–C). This experiment was repeated in acetate proline medium, a condition of low carbon and nitrogen catabolite repression. In this case, *MLS1* mRNA

Table 2. *DAL7* and *MLS1* mRNA levels in cells provided with various carbon sources

Gene	Growth Conditions			
	YPD	YPE	YPAc	YPOA
<i>DAL7</i>	0.08	0.09	0.08	0.11
<i>MLS1</i>	0.05	1.0	1.78	0.64
<i>ACT1</i>	1.0	1.0	1.0	1.0

Yeast cultures (strain GA1-8C) were grown in liquid culture, RNA was isolated, separated by electrophoresis and blotted onto Nylon filters as described in Materials and Methods. DNA fragments used for hybridization were the 3.5 kb *Hind*III, 2.5 kb *Hind*III, and 3.5 kb *Tam*HI-*Eco*RI fragments containing *MLS1*, *DAL7*, and actin gene sequences, respectively. These fragments were labelled as described in Materials and Methods. The areas of the corresponding bands were scanned with a Hirshmann Elscipt 400 gel scanner. The relative band intensities are listed in the table.

was produced at high levels, but *DAL7* mRNA could not be detected (Figure 5, lanes F and D). *MLS1* mRNA production was only very modestly sensitive to nitrogen catabolite repression (Figure 5, lanes F and G; note the difference in the *TCM1* mRNA signals) and displayed a small response to addition of OXLU to the medium (Figure 6, lanes A and B). For unknown reasons, the small response of *MLS1* expression to inducer observed with strain Σ 1278b (Fig. 6, lanes A and B) was below detection when strain GA1-8c was used (Fig. 5, lanes H and I). Measurement of *MLS1* response to OXLU induction was not performed with acetate provided as carbon source because acetate medium possesses a pH of 6.0, a pH which does not permit transport of oxalurate into the cell (41). Disruption of the negative regulatory element of the allantoin pathway genes, *DAL80* (42), had no effect on *MLS1* expression (Figure 6, lanes A and C). More quantitative assessment of *MLS1* expression when cultures were provided with various carbon sources is shown in Table 2. Steady state levels of *MLS1* mRNA were highest when acetate was provided as carbon source (YPAc). They were 44% and 64% lower in ethanol (YPE) and oleic acid (YPOA) media, respectively (Table 2).

Growth characteristics of *mls1* and *dal7* null mutants

The open reading frame encoding the *MLS1* malate synthase was eliminated by replacing it with the *LEU2* gene as described in Methods. A *DAL7* gene null mutation was produced by disruption at amino acid position 5 (8). Using these constructs, single *mls1* and *dal7* null mutants as well as a *mls1*, *dal7* double mutant strain were constructed as described in Methods. The three null mutant strains did not produce either *MLS1* and/or *DAL7* mRNA as expected from their genotypes (data not shown). However, all strains grew equally well in media containing YPD and different carbon sources such as ethanol, acetate, or oleate (data not shown). On synthetic media differences were more pronounced, although all except one of the strains were able to grow more or less well in either ethanol or acetate medium (Figure 7). Deletion of the *MLS1* gene exhibited the strongest phenotype (Figure 7). Disruption of *DAL7* alone had no detectable effect on growth with acetate or ethanol provided as carbon source, which was not unexpected since we could not detect *DAL7*-specific mRNA in cells cultured with these carbon sources. These results were

Figure 7. Growth curves of wild type, *mls1*, and *dal7* single null mutant, and *mls1/dal7* double null mutant strains. The experiment was performed as described in methods.

confirmed when malate synthase enzyme activity was determined in cells provided with various carbon sources. As shown in Table 3, there was significant malate synthase activity in the wild type strain with ethanol or acetate as carbon source. The enzyme levels were substantially lower in the null mutant strains except for the *dal7* disruption mutant grown with acetate as carbon source, a condition known to result in high levels of *MLS1* mRNA. Why similarly high levels of enzyme activity were not observed in this strain with ethanol as carbon source is not understood.

The above results suggested that *S.cerevisiae* likely contains more than two genes encoding malate synthase proteins. Consistent with this suggestion is the observation that when autoradiographs of Northern blots hybridized with a *DAL7*-specific probe were over exposed, a second RNA species with a slower mobility than the *DAL7* transcript was observed (Figure 8). This species responded to addition of OXLU to the culture and in other experiments we have observed a similar sized, *DAL7*-hybridizable mRNA species to respond to disruption of the *DAL80* locus (J. Daugherty and T.G. Cooper, unpublished results).

Figure 8. Identification of an unknown transcript cross-hybridizing with the *DAL7* gene. Wild-type strain $\Sigma 1278b$ was grown in YNB minimal medium. The carbon source was 2% glucose and the nitrogen source was 0.1% proline (PRO). Where OXLU was used (PRO+), it was provided at a final concentration of 0.25 mM. 7 μ g of poly (A)⁺ RNA were loaded per lane. The probe was an isolated 2.5 kb random primed-labelled *Hind*III fragment from plasmid pHY3 containing the *DAL7* gene. Note a small and difficult to observe transcript below the *DAL7* transcript. It derives from the *DCG1* gene which was also contained on the hybridization probe we used.

Figure 9. Ureidoglycollate hydrolase activity in isogenic wild type (W303-1a) and *dal7* disrupted strains (HY40-5 and HY39-11). The strains were grown in Wickerham's minimal glucose proline medium.

Requirement of malate synthase for optimal operation of the ureidoglycollate hydrolase reaction

The function of malate synthase in allantoin metabolism is to remove toxic glyoxylate, a product of the ureidoglycollate (UG) hydrolase reaction, from the cell (5, 7). Since the reaction catalysed by UG hydrolase is quite freely reversible, removing glyoxylate from the vicinity of the enzyme will increase the rate

Table 3. Malate synthase activity in wild type, *mls1* deletion, and *dal7* disruption mutants growing on various carbon sources

Mutation (strain)	YPE		YPAc		YPD (4%)	
none (GA1-8C)	119		165		2.2	
	132	145 +/- 38	132	144 +/- 20	0.6	1.0 +/- 1.0
	183		135		0	
<i>mls1</i> Δ (JR85)	6.8		4.6		2.0	
	12.5	8 +/- 4	3.2	4 +/- 1	2.1	1.4 +/- 1.4
	5.0		3.0		0	
<i>dal7::HIS3</i> (JD1)	31		141		4.4	
	10	17 +/- 14	124	145 +/- 26	2.4	3.0 +/- 1.4
	11		171		2.2	
<i>mls1</i> Δ, <i>dal7::HIS3</i> (JR86)	13		3.0		0	
	18	20 +/- 10	0.4	1 +/- 1	0	0.4 +/- 0.4
	30		0		1.0	

Yeast cultures (strain GA1-8C) were grown in liquid culture and harvested after 24 hrs. of growth as described in Materials and Methods, except that the glucose concentration in YPD was 4% instead of 2%. Enzyme activities are given in micromoles/min./mg protein. Also shown are the median and range values of these measurements.

of ureidoglycollate hydrolysis. Conversely, disruption of the *DAL7* gene and loss of malate synthase activity would be expected to result in increased intracellular levels of glyoxylate which would in turn be expected to slow the reversible UG hydrolase reaction. To ascertain whether or not this expectation occurred experimentally, we measured ureidoglycollate hydrolase activity in wild type and *dal7* disruption mutants. As shown in Figure 9, ureidoglycollate hydrolase activity was diminished by three to four fold in the disruption strain.

DISCUSSION

The work presented here demonstrate that *S. cerevisiae* contains at least one gene, in addition to *DAL7*, that encodes malate synthase (*MLS1*). The deduced *MLS1* protein sequence allows the conclusion that the protein encoded by this open reading frame is indeed a malate synthase. This conclusion was supported by the experimental result that malate synthase activity increased by a factor of 15 in transformants containing the 6.8kb *EcoRI*-fragment on the multi-copy plasmid YEp352 (43, data not shown), and decreased when the *MLS1* gene was deleted.

Regulation of the *MLS1* and *DAL7* genes is quite distinct. *MLS1* expression is regulated as one would expect of a gene that participates in catabolism of poor carbon sources, i.e. it is highly sensitive to carbon catabolite repression and is nearly insensitive to the nitrogen source provided. This kind of regulation can be expected for a component of the glyoxylate cycle. On the other hand, *DAL7*, which participates in the catabolism of allantoin, is insensitive to carbon catabolite repression, but highly sensitive to nitrogen catabolite repression. An interesting aspect of the regulation that we did not pursue is the observation that *DAL7* is so poorly expressed in cells provided with acetate as sole carbon

source. This phenomenon may represent a control mechanism that prevents *DAL7* expression in some way when cells produce high malate synthase levels from other genes. The modest response of *MLS1* to the allantoin pathway gratuitous inducer, OXLU, and nitrogen catabolite repression is probably accounted for by the fact that its 5' flanking region contains multiple sequences that are homologous to *UAS_{NTR}*, the cis-acting element demonstrated to be the one through which nitrogen catabolite repression is exerted, and one sequence homologous to the *DAL7 UIS*, the cis-acting element required for response of *DAL* gene expression to inducer (8).

The results of this work raise the possibility that *S. cerevisiae* contains at least one and perhaps two more genes encoding malate synthase. This suggestion is based on the observations that malate synthase activities can be measured in the *mls1*, *dal7* double null mutants and that these double null mutants were able to grow in medium containing either ethanol or acetate as sole carbon source and ammonium sulfate as nitrogen source. Further consistent with the possibility of additional malate synthase genes, we obtained preliminary evidence for existence of a transcript with sufficient homology to *DAL7* as to weakly hybridize to *DAL7*-specific probes. If this transcript is produced when cells are provided with either glucose or acetate, a situation for which we have no evidence at present, existence of this single gene product would account for the growth and malate synthase activities observed in the *mls1*, *dal7* null mutants. On the other hand, since this transcript displays at least some of the characteristics of *DAL7* expression, it may not be produced in acetate medium. In that case, one would be forced to suggest the existence of two additional genes to account for the growth and enzyme data we reported. Consistent with this suggestion are the observations of McCammon and his colleagues who have

isolated two classes of mutants which grow poorly in acetate ammonia medium and exhibit severely decreased levels of malate synthase activity. One class of mutants contains defects which fail to complement *mls1* mutations and are hence allelic with *mls1*. The second class of mutations effectively complements *mls1* mutations and are hence distinct (M.T. McCammon and J.M. Goodman, personal communication).

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