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Two structural genes are encoding malate synthase isoenzymes in *Saccharomyces cerevisiae*

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We report on the isolation of a gene encoding yeast malate synthase. A yeast genomic library was screened using a probe homologous to the yeast enzyme obtained by the polymerase chain reaction. The nucleotide sequence of the cloned gene was determined. Computer analysis showed that the isolated gene is identical to the one previously described as *DAL7*, which is involved in allantoin metabolism [Mol. Cell. Biol. 9 (1989) 3231–3243]. Enzymatic activities of multicopy transformants, Southern analysis and disruption mutants predict the existence of two genes encoding malate synthases that are differentially regulated at the transcriptional level.

Malate synthase; Glyoxylate pathway; Allantoin metabolism; Gene disruption.

1. INTRODUCTION

Utilization of ethanol by *Saccharomyces cerevisiae* requires the presence of the glyoxylate pathway that prevents the net loss of the ethanol carbon atoms as carbon dioxide in the Krebs cycle. Isocitrate lyase and malate synthase are the key enzymes of this pathway.

Malate synthase has been purified from bakers' yeast. It is composed of three identical subunits with a M_r of 66,000 Da each [1]. The synthesis of the enzyme is induced by ethanol and repressed by glucose [2]. Genes encoding malate synthase have been cloned and sequenced from different organisms including *Escherichia coli* [3,4], *Hansenula polymorpha* [5], *Neurospora crassa* [6,7] and *Aspergillus nidulans* [8,7].

In order to characterize the glyoxylate pathway in *S. cerevisiae* we tried to isolate mutants in the genes encoding isocitrate lyase and malate synthase, screening for their inability to grow on ethanol but retaining growth on pyruvate. One type of mutants lacked isocitrate lyase activity and was used for the isolation of the respective gene [9]. No mutants affected in the malate synthase structural gene could be obtained. We therefore used here a different strategy to clone the gene. In addition our results indicate that *S. cerevisiae* contains two genes encoding malate synthase isoenzymes. During the preparation of the manuscript the sequence of a possible candidate for a second gene being homologous but not identical to ours, called *MLS1*, has been entered in the EMBL-database by C. Hartig. For this reason, the gene described in this paper was named *MLS2*.

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2. MATERIALS AND METHODS

2.1. *Strains*

S. cerevisiae AMW-13C$^+$ (*MATa trp1*(fs) *ura3*(fs) *leu2–3,112 his3–11,15 can1*; where 'fs' stands for frameshift mutation) was used as recipient for transformation experiments and deletion studies.

Double stranded and single stranded DNAs were isolated in the *E coli* strains XL1-Blue and HB101.

2.2. *Media, growth conditions and enzymatic analysis*

Media and growth conditions were as described in [9] Malate synthase activity was assayed according to [10]. For preparations of crude extracts cells were grown overnight on 0.67% Difco yeast nitrogen base without amino acids with 2% glucose, supplemented with amino acids, adenine and uracil as required. Leucine was omitted for growth of transformants. The cells were transferred to 0.67% yeast nitrogen base without amino acids and ammonium sulfate with either 5 g/l ammonium sulfate and 3% ethanol or 10 mM urea and 2% glucose to allow the induction of the respective malate synthase isoenzymes. The amino acids and nitrogen bases esential for the growth of each particular strain were also added.

2.3. *Isolation of the MLS2 gene*

The gene encoding malate synthase from *S cerevisiae* was cloned by screening the genomic library constructed by Nasmyth and Tatchell in the multicopy vector YEp13 [11]. As a probe we used a DNA fragment homologous to the malate synthase gene prepared by the polymerase chain reaction (PCR). Thus, the nucleotide sequence of malate synthases from several organisms was compared. The peptides 'AMGGMAA' and 'MEDAATA' corresponding to the positions 326 and 451 in the *E coli* enzyme, were present in almost all the enzymes. Two mixtures of degenerated oligonucleotides, 5'-GC-NATGGGNGGNATGGCNGC-3' and 5'-GCNGTNGCNGCG/ATCT/CTCCAT-3' corresponding to the peptides (where N stands for any deoxynucleotide) were prepared. Total chromosomal DNA obtained from the strain AMW-13C$^+$ was used as a template. The DNA was amplified using Taq-DNA-polymerase from Beckman (Germany)

with 25 cycles of 1.5 min at 92°C, 2 min at 60°C and 3 min at 72°C, using the buffer conditions according to the protocol of Perkin-Elmer/Cetus. As expected, an about 380 bp fragment was obtained after PCR amplification.

2.4. *Nucleic acid preparations, hybridization experiments and sequencing*

All DNA manipulations were as described previously [9].

3. RESULTS AND DISCUSSION

3.1. *Cloning of the MLS2 gene*

A recombinant plasmid containing the *MLS2* gene was isolated by screening a genomic library in *E. coli* with the PCR-generated probe as described in section 2. From about 5,000 colonies tested only one hybridized to the probe. The restriction map of the recombinant plasmid (pMLS1) isolated from this colony is shown in Fig. 1A. The gene was located within the DNA insertion by Southern analysis using the above PCR-generated probe after digestion of the plasmid with different restriction endonucleases. The probe hybridized to a 1.7 kb *Stu*I-*Hin*dIII fragment (data not shown). Therefore, we sequenced this DNA fragment and its flanking regions.

The deduced amino acid sequence of the open reading frame found was compared to the EMBL data bank. Unexpectedly, the cloned gene turned out to be identical to the *DAL7* gene involved in the allantoin metabolism [12]. In addition the deduced amino acid sequence shows high similarity to malate synthases from other organisms (Fig. 2).

3.2. *Overproduction of malate synthase from plasmid pMLS1*

The comparison of the amino acid sequences of the *MLS2* gene product and malate synthases from other organisms suggests that the isolated gene (=*DAL7*) encodes a malate synthase. If it indeed encodes a functional protein, yeast cells transformed with the multicopy plasmid pMLS1 should contain higher specific enzymatic activities than a non-transformed strain on media where *DAL7* is expressed.

The transcription of the *DAL7* gene shares the regulation of most of the enzymes of the allantoin pathway [12], i.e. they are sensitive to nitrogen catabolite repression and induced by allophanate and other related metabolites such as urea [13]. On the other hand, malate synthase has been described as a glyoxylate pathway enzyme that is synthesized in the presence of ethanol but is repressed by glucose [2].

When ethanol was used as a carbon source, under repressing conditions for the allantoin pathway isoenzyme (i.e. in the presence of ammonium sulfate), the levels of malate synthase activities were similar in the transformed and the untransformed strain (Table I). However, when urea was added as an inducer and glucose was used as a carbon source, an about 4-fold increase in specific malate synthase activity was found in the strain carrying the plasmid pMLS1. This clearly indicates that pMLS1 encodes the malate synthase isoenzyme involved in the allantoin pathway and cannot be repressed by a fermentable carbon source.

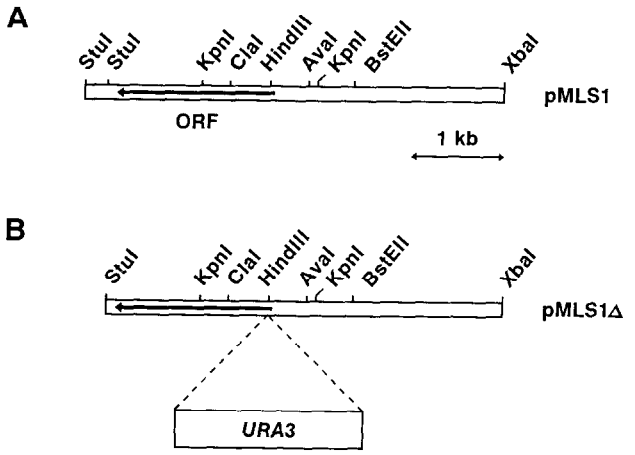

Fig. 1. (A) Restriction map of the genomic DNA-fragment containing the *MLS2* gene. (B) Restriction map of plasmid pMLS1# The *Stu*I-*Xba*I fragment containing almost the entire insertion of pMLS1 was cloned into the vector pUK21 [15]. For disruption of the chromosomal copy of *MLS2*, the *URA3* gene was inserted into the unique *Hin*dIII.

3.3. *Identification of a second homologous gene by Southern analysis*

The results presented above suggest the presence of two malate synthase isoenzymes in *S. cerevisiae*. To confirm this hypothesis a Southern analysis was performed (Fig. 3). Yeast chromosomal DNA digested with *Xba*I, *Hin*dIII and *Bam*HI hybridized to the PCR-generated probe gave two bands where only one was expected from the restriction map of pMLS1. When the 1.7 kb *Hin*dIII-*Stu*I fragment of plasmid pMLS1 (see Fig. 1) was used as a probe only one band was observed. Thus the PCR-generated probe corresponds to a highly conserved domain of both malate synthases and gives

Table I

Specific activities of malate synthase in a wild-type strain and a multicopy transformant.

Strain	Plasmid	Specific activity (mU/mg)	
		SCE	SDU
AMW-13C$^+$	–	389	87
AMW-13C$^+$	pMLS1	386	341

To assay the malate synthase activity of the glyoxylate pathway, cells were grown on synthetic medium with ammonium sulfate as a nitrogen source and ethanol as a carbon source (SCE). To distinguish the malate synthase isoenzyme of the allantoin pathway, cells were grown on synthetic medium with urea as a nitrogen source and glucose as a carbon source (SDU).

```
                    1                                                                                                  100
S.cerevisiae (DAL7) .........M VKISLDNTAL YADIDTTPQF EPSKTTVADI LTKDALEFIV LLHRTFNSTR KQLLANRSNL QSKLDSGEYR FDFLPETEQI RNDPTWQG.A
A.nidulans          .......MSQ VDAQLKDVAI LGSVSNEA.. .......RKI LTKEACAFLA ILHRTFNPTR KALLQRRVDR QAEIDKGHLP .DFLPETKHI RDDPSWKG.A
N.crassa            .......MAS VETLLQGVTI SGPIEEHQ.. .......RKI LTPQALSFVA LLHRSFNQTR KNLLERRHVR QAEIDRGVLP .DFLPETKHI RENPTWKGAA
H.polymorpha        ......MAQI NPTTLDNVKI LGEVSDKPLL ..SKATPRDI LTKDALKFIV LLHRSFNETR KQLLENRQKV QERLDAGE.S LHFLEETKYI REDPNWKC.L
C.maxima            MGSLGMYSES AVRKK.SSRG YDVPEGVDIR GRYDEEFARI LNKEALLFVA DLQRTFRNHI RYSMECRREA KRRYNEGAVP GFD.PATKYI RESE..WTCA
C.sativus           MGSLGMYSES GLTKKGSSRG YDVPEGVDIR GRYDEEFAKI LNKEALLFIA DLQRTFRNHI KYSMECRREA KRRYNEGGLP GFD.PATKYI RDSE..WTCA
G.hirsutum          MIGLGSYGYT APSSKKIN.A YDVPQGVDIR GRFDEEFAKI LTKDALQFVA DLQREFRNHI KYAMECRKEA KRRYNEGALP GFD.PATRYI REGK..WTCV
R.communis          .MRYDTYGDS APIKKTGA.G YDVPEGVDIR GRYDGEFAKI LTRDALQFVA DLQREFRNRI RYAIECRKEA KSRYNAGALP GFEHPATKYI REGE..WTCA
B.napus             ......MELE TSVYRPNVAV YDSPDGVEVR GRYDQVFAKI LTRDALGFVA ELQREFRGHV RYAMECRREV KRRYNSGAVP GFD.PSTKFI RDGE..WVCA
E.coli              .......... ......MTEQ ATTTDELAFT RPYGEQEKQI LTAEAVEFLT ELVTHFTPQR NKLLAARIQQ QQDIDNGTLP DF.ISETASI RDAD..WKIR

Consensus           mgslgmyses ap-kk--v-- ydvpegvdir grydeefakI LTk-Allfva dLqRtFrnhi kyl-ecRrea krryneGalp gfdlP-TkyI Re-etwwtca

                    101                                                                                                200
S.cerevisiae (DAL7) IPAPGLINRS SEITGPPLRN MLVNDLNAEV TTYMTDFEDS SSPTWENMIY GQVNLYDAIR NQIDF..KTP RKEYRLKGDI SRLPTLIVRP RGWHMVEKHL
A.nidulans          PPAPGLVDRR VEITGPTDRK MVVNALNSDV WTYMADFEDS SAPTWDNMIN GQINLYDAIR RQVDF..KQG QKEYKLRTD. RTLPTLIARA RGWHLDEKHF
N.crassa            PAAPPLVDRR VEMTGPTDRK MVVNALNSDV YTYMADFEDS SAPTWANMVN GQVNLYDAIR RQIDF..KQG PKEYKLRTD. RTLPTLIVRP RGWHLEEKHV
H.polymorpha        PTHPKLQCRK IEITGPPDAK MIVNAFNTDV FTYMTDFEDS CSPTWNNMIY GQVNLYDAIR DRIDFTNEAT GKRYKINREG RRVPVMIVRP RGWHMVDKHI
C.maxima            SVPPAVADRR VEITGPVERK MIINALNSGA KVFMADFEDA LSPNWENLMR GQINLKDAVD GTISFHDKAR NKVYKLNDQ. ..TAKLFVRP RGWHFAEAHI
C.sativus           PVPPAVADRR VEITGPVERK MIINALNSGA KVFMADFEDA LSPNWENLMR GQINLKDAVD GTISFHDRVR NRVYKLNDR. ..TAKLFVRP RGWHLPEAHI
G.hirsutum          PFPPAVADRR VEITGPVERK MIINALNSGA KVFMADFEDA LSPSWENLMR GQINLKDAVE GTITFNDKAR NRVYKLNNE. ..IAKLFVRP RGWHLPEAHI
R.communis          PVPPAVADRK VEITGPVERK MIINALNSGA KVFMADFEDA LSPSWENLMR GQVNLRDAVN GTISFHDKAR NRVYKLNDQ. ..IAKLFVRP RGWHLPEAHI
B.napus             SVPPAVADRR VEITGPVERK MIINALNSGA KVFMADFEDA LSPSWENLMR GQVNLKDAVD GSITFNDKAR NKVYKLNDQ. ..VAKLFVRP RGWHLPEAHI
E.coli              GIPADLEDRR VEITGPVERK MVINALNANV KVFMADFEDS LAPDWNKVID GQINLRDAVN GTISYTNEA. GKIYQLKPN. ..PAVLICRV RGLHLPEKHV

Consensus           pvpPavadRr VEITGPveRK MiiNALNsga kvfMaDFEDa lsPtWeNlmr GGiNLkDAvd gtIsFhdkar nkvYKLndd- r--akLfVRP RGWHlpEaHi

                    201                                                                                                300
S.cerevisiae (DAL7) YIDDEPISAS IFDFGLYFYH NAKEL...VK IGKGPYFYLP KMEHHMEVKL WNDIFCVAQD FIGMPRGTIR ATVLIETLPA AFQMEEIIYQ IREHSSGLNC
A.nidulans          TVDGEPISGS LFDFGLYFFH NAKEL...VA RGFGPYFYLP KMESHLEARL WNDVFNLAQD YIGMPRGTIR GTVLIETITA AFEMEEIIYE LRDHSSGLNC
N.crassa            TIDGEPVSGS LFDFGLYFFH NAKEL...VQ RGFGPYFYPP KMESHLEARL WNDAFNLAQD YVGIPLSTIR GTVLIETITA AFEMDEIIFE LRNHTSGLNR
H.polymorpha        LVDGEPISAS IMDFGLFFFH NAKFL...LS QGLAPFFYLP KMEHYKEAQL WNDIFCVAQD CLEIPRGTIK ATVLIETLPI SYQLDEVLYA LRDHSAGLNC
C.maxima            FIDGEPATGC LVDFGLYFFH NHANFRRSQG QGSGPFFYLP KMEHSREAKI WNSVFERAEK MAGIERGSIR ATVLIETLPA VFQMDEILYE LRDHSVGLNC
C.sativus           FIDGEPATGC LVDFGLYFFH NHANFRRSQG QGYGPFFYLP KMEHSREAKI WNSVFERAEK MAGIERGSIR ATVLIETLPA VFQMNEILYE LRDHSVGLNC
G.hirsutum          FIDGEPATGC LVDFGLYFYH NYATFRNTQG QGFGPFFYLP KMENSREAKI WNSVFEKAEK MAGIEKGSIR ATVLIETLPA VFQMDEIFYE LRDHSVGLNC
R.communis          LIDGEPATGC LVDFGLYFYH NYAAFRRNQG AGYGPFFYLP KMEHSREAKI WNCVFEKAEK MAGIERGSIR ATVLIETLPA VFQMNEILYE LRDHSVGLNC
B.napus             LIDGEPATGC LVDFGLYFFH NYAKFRQTQG SGFGPFFYLP KMEHSREAKI WNSVFERAEK MAGIERGSIR ATVLIETLPA VFQMNEILYE LRDHSVGLNC
E.coli              TWRGEAIPGS LFDFALYFFH NYQAL...LA KGSGPYFYLP KTQSWQEAAW WSEVFSYAED RFNLPRGTIK ATLLIETLPA VFQMDEILHA LRDHIVGLNC

Consensus           fiDGEPatGc LvDFGLYFfH N-a-frrsqg qGfGPfFYLP KMEhsrEAki WN-vFerAek maGieRgsIR ATVLIETLPA vFQMdEilYe LRDHSvGLNC

                    301                                                                                                400
S.cerevisiae (DAL7) GRWDYIFSTI KKLRNLNEHV LPNRDLVTMT SPFMDAYVKR LINTCHRRGV HAMGGMAAQI PIKDDPKANE AAMNKVRNDK IREMKNGHDG SWVAHPALAP
A.nidulans          GRWDYIFSFI KKFRQHPNFV LPDRSDVTMT VPFMDAYVKL LIKTCHKRGV HAMGGMAAQI PIKDNAEAND KAMEGVRADK LREVRAGHDG TWVAHPALAS
N.crassa            GGWDYIFPFI KEVRRFPNFV LPDRSDVTMT VPFMEAYVKL LIKTLHRLVV HAM.GMAAQI PIKDDKAAND KAMEGVRADK LREARAGHDG TWVAHPALAS
H.polymorpha        GRWDYMFSTI KRLRNQKQHI LPDRHQVTMT VPFMTNYVKQ LIKICHKRGV HAMGGMAATI PIKDDPEKNA AAMEAVRQDK LREVLAGHDG TWIAHPGLLP
C.maxima            GRWDYIFSYV KTFQAHLDRL LPDRVQVGMA QHFMRSYSDL LIRTCHTVVC H.VGGMAAQI PIRDDPKANE MALELVRKDK LREAKAGHDG TWAAHPGLIP
C.sativus           GRWDYIFSYV KTFQAHPDRL LPDRVLVGMT QHFMRSYSDL LIRTCHRRGV HAMGGMAAQI PIRDDPKANE VALELVRKDK LREVKAGHDG TWAAHPGLIP
G.hirsutum          GRWDYIFSYV KTFQGHPDRL LPDRGQVGMT QHFMRSYSDL LIRTCHRRGV HAMGGMAAQI PIRDDPTANE AAFELVRKDK QREVKAGHDG TWAAHPGLIK
R.communis          GRWDYIFSYV KTFQAHPDRP LPDRVQVGMT QHFMKSYSDL LVWTCHRRGV HAMGGMAAQI PIRDDPAANK AALELVRKDK LREVKAGHDG TWAAHPGLIP
B.napus             GRWDYIFSYV KTFQAHPDRL LPDRVLVGMG QHFMRSYSDL LIRTCHKRGV HAMGGMAAQI PIRDDPKANE MALDLVKKDK LREVRAGHDG TWAAHPGLIP
E.coli              GRWDYIFSYI KTLKNYPDRV LPDRQAVTMD KPFLNAYSRL LIKTCHKRGA FAMGGMAAFI PSKDEEHNNQ V.LNKVKADK SLEANNGHDG TWIAHPGLAD

Consensus           GRWDYIFSyv Ktfqahpdr- LPDRvqVgMt qhFMrsYsdl LIrTCHrRGV HAMGGMAAQI PIrDDpkANe aAlelVRkDK lREvkAGHDG TWaAHPgLip

                    401                                                                                                500
S.cerevisiae (DAL7) ICNEVF.SNM G.TANQIYFV P.D..VHVTS SDLLNTKIQD AQVTTEGIRV NLDIGLQYME AWLRGSGCVP INHLMEDAAT AEVSRCQLYQ WVKHGVVLSD
A.nidulans          IASEVFNKYM P.TPNQMHVR RED..VNITA NDLLNTNVP. GKITEDGIRK NLNIGLSYME GWLRGVGCIP INYLMEDAAT AEVSRSQLWQ WARHGVTTSE
N.crassa            IALEVFNKHM P.TPNQLFNR RED..VKIGQ QDLLNMNVP. GSSTEDGIRK NLNTGLGYTE PWIRGVGCVP IKHPQEDAAT AEVSRSQLWQ WVKHRVTTAE
H.polymorpha        TALSVFQEHM P.TPNQIHVQ K.N..VEITE ADLVDTNIPD GKITMKGVSA NIYIGLNYME SWLRGLGCVP INNLMEDAAT AEVSRLQLYS WCKHAVKMDD
C.maxima            ACMEVFTNSM GNAPNQIRSA RRDDAANLTE DDLLQQ..PR GVRTLEGLRL NTRVGIQYLA AWLTGTGSVP LYNLMEDAAT AEISRVQNWQ WLKYGVELDG
C.sativus           ACMEVFTNNM GNAPNQIRSM RRDDAANLTE EDLLQQ..PR GVRTMEGLRL NTRVGIQYLA AWLTGAGSVP LYNLAEDAAT AEISRVQNWQ WLKYGVELDG
G.hirsutum          TCMEVFTNNM GNTPNQIETV KRDYASNLTE DDLLQR..PR GVRTMEGLRL NTRVGIQYLA AWLTGSGSVP LYNLMEDAAT AEISRVQIWQ WLKYGVELDG
R.communis          ACMEVFANNM GNTPHQIQAM KREDAANITE EDLIQR..PR GVRTLEGLRL NTRVGIQYLA AWLTGTGSVP LYNLMEDAAT AEISRVQNWQ WLKYGVELDG
B.napus             ICMDAFSH.M GNNPNQIKSM KRDDASAITE EDLLQI..PR GVRTLEGLRL NTRVGIQYLA AWLTGSGSVP LYNLMEDAAT AEISRVQNWQ WIRYGVELDG
E.coli              TAMAVFNDIL GSRKNQLEVM REQDAP.ITA DQLLAP..CD GERTEEGMRA NIRVAVQYIE AWISGNGCVP IYGLMEDAAT AEISRTSIWQ WIHHQKTLSN

Consensus           -cmeVFtnnM gntPNQirsm rrdda-niTe dDLLq-n-Pr GvrTleGlRl NtrvGiQYla aWLtG-GsVP lynLMEDAAT AEiSRvQNWQ WlkygVeldg

                    501                                                                                    580
S.cerevisiae (DAL7) T..GDKVTPE LTAKILNEET AKLASASPLG EKNK..FALA AKYFLPEVTG KI.FSDFLTT LLYDEIIKPS AKPVDLSKL.
A.nidulans          ...GKKVDKA YALRLLKEQA DALAAKGPKG ..NK..FQLA GRYFSEQVTG E.DYADFLTS LLYNEISSPG ....TASKL.
N.crassa            ...GKHVDKR YPLKLLKEAD RQRLAKAPQG ..NK..FNLA AQYFASQVTG E.DYADFLTC LLYNEITSAG .NSLPASKL.
H.polymorpha        T..GKTITPE FISKLIDEEA ERCAANKP.. .NNK..FKIA ADCLKKEING HTEVAEFLTT LLYDDIVTIG PE.VDISSLK
C.maxima            DGLGVRVNKE LFARVVEEEM ERIEREVGKE KFRKGMYKEA CKMFTRQCTA P.TLDDFLTL DAYNHIVIHH PRELSRL...
C.sativus           DGLGVRVNKE LFGRVVEEEM ERIEREVGKE RFKKGMYKEA CKMFTRQCTA P.NLDDFLTL DAYNYIVIHH PRELSKL...
G.hirsutum          DGLGVRVN.H VFGRVVEEEM ARIEREVGKE KFKKGMYKEA CKIFTRQCTA S.TLDDFLTL DAYNYIVIHH PKDVSSKL..
R.communis          DGLGVKVTFD LLGRVVEDEM ARIEREVGKE KFKKGMYKEA CKMFVRQCAA P.TLDDFLTL DAYNNIVIHY PKG.SSRL..
B.napus             DGLGVRVSKE LFGRVVEEEM ERIEKEVGKD KFKRGMYKEA CKMFTKQCTA A.ELDDFLTL AVYDHIVAHY PINASRL...
E.coli              ...GKPVTKA LFRQMLGEEM KVIASELGEE RFSQGRFDDA ARLMEQITTS D.ELIDFLTL PGYRLLA... .........

Consensus           dglGvrV-ke lfgrvveEEm erierevgke kf-KgmykeA ckmftrqcTa p-eldDFLTl daYneIvihh p-elsr-kl-
```

Fig. 2. Comparison of malate synthases from different organisms. The amino acids sequences were obtained from: *S cerevisiae* [12 and our data], *A nidulans* [8], *N crassa* [8], *H polymorpha* [5], *Cucurbita maxima* [16], *Cucumis sativus* [17], *Gossypium hirsutum* [18], *Ricinus communis* [19], *Brassica napus* [20] and *E. coli* [4]. Capital letters in the sequences designate amino acids identical in at least 8 malate synthases from different organisms.

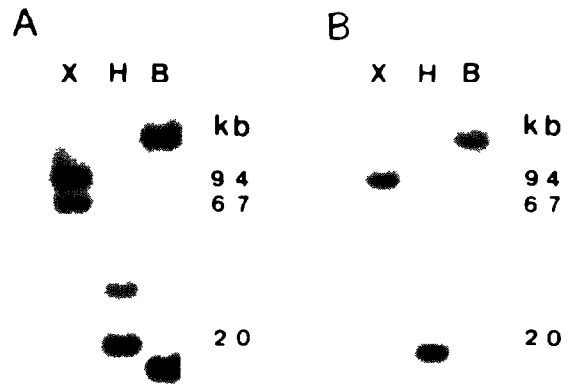

Fig. 3. Southern analysis. A. Genomic DNA was digested with *Xba*I (X), *Hin*dIII (H) and *Bam*HI (B) and hybridized with the PCR-generated probe (see section 2). B Genomic DNA digested with the same enzymes was hybridized to the 1.7 kb *Stu*I-*Hin*dIII fragment of pMLS1.

strong hybridization signals, whereas the flanking regions contained in the pMLS1 probe are less homologous and lead to a reduced cross-hybridization.

3.4. *Disruption of the MLS2 gene*

Further evidence for the presence of two genes encoding malate synthase was obtained by constructing a disruption mutant in the *MLS2* gene. For this purpose, plasmid pMLS1# was obtained by inserting the *URA3* gene into the *Hin*dIII site at the beginning of the open reading frame of the *MLS2* gene (Fig. 1B). *Cla*I and *Bst*EII were used to digest the plasmid prior to transformation of the haploid strain AMW-13C$^+$. A transformant carrying the *MLS2* gene disrupted by the *URA3* marker was identified by Southern analysis (data not shown). This mutant strain grows on ethanol as a sole carbon source and produces malate synthase activity. However, no malate synthase activity was detected when urea was used as an inducer on glucose media, as expected for a disruption in the gene encoding the isoenzyme of the allantoin pathway.

3.5. *Transcriptional regulation of the genes encoding the malate synthase isoenzymes*

The transcriptional regulation of the malate synthase genes was studied by Northern analysis. The expression of the *DAL7* (=*MLS2*) gene in relation to the allantoin pathway has been studied by others, previously [12]. Here we prepared total RNA from cells grown on media containing urea as a nitrogen and glucose as a carbon source. In a Northern blot, this RNA was hybridized to the *Stu*I-*Hin*dIII fragment of pMLS1 (Fig. 4A). On the other hand, to study the regulation of the isoenzyme functioning in the glyoxylate pathway, RNA from cells grown on different carbon sources in the presence of ammonium sulfate (i.e. under repressing conditions for *DAL7*) was hybridized to the PCR-generated probe (Fig. 4B). In the first case, a hybridization signal was obtained for the malate synthase gene in the wild-type strain but was absent in its disruption derivative. We expect the gene encoding the glyoxylate pathway isoenzyme to be repressed under these growth conditions by the presence of glucose. In the second case, a strong band was found when cells were grown on ethanol but not on glucose media. Only a weak band was detected in the glycerol preparation. In addition, no mRNA was detected in yeast strains carrying mutations in the *CAT1* or the *CAT3* genes (data not shown). Such mutants are defective in derepression of gluconeogenic and glyoxylate enzymes, including malate synthase, after growth on glucose [14].

The results described in this paper show that *S. cerevisiae* contains two malate synthase isoenzymes. For the utilization of ethanol as a sole carbon source, yeast utilizes the glyoxylate pathway with isocitrate lyase and malate synthase as the key enzymes. Thus, one malate synthase isoenzyme, which is repressed by glucose and expressed on ethanol media, will be used in this pathway and not be susceptible to the control mechanisms of the allantoin pathway. On the other hand, we have shown above, that *DAL7* (*MLS2*) is repressed when ammonium sulfate is present in the medium. A common denominator of the glyoxylate and the allantoin pathways

Fig. 4. Northern blot analysis. (A) Total RNA was prepared from cells of strain AMW-13C$^+$ grown on synthetic minimal medium with urea as a nitrogen source (wt) and glucose as a carbon source, as well as of the *mls2* disruption derivative of this strain grown in the same conditions (d). As radioactive probes a mixture of the 1.7 kb *Stu*I-*Hin*dIII fragment from pMLS1 and a DNA fragment containing the yeast actin gene were used. (B) Total RNA was prepared from cells of strain AMW-13C$^+$ grown on rich media (containing ammonium sulfate) with ethanol (E), glycerol (G) and glucose (D) as carbon sources. As probes a mixture of the PCR-generated fragment and a DNA fragment containing the yeast actin gene were used.

is the formation of glyoxylate. It seems likely that the isoenzyme encoded by *DAL7* (*MLS2*) is used for further metabolizing the glyoxylate formed in the latter pathway to avoid its accumulation.

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