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Targeting of malate synthase 1 to the peroxisomes of *Saccharomyces cerevisiae* cells depends on growth on oleic acid medium

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The eukaryotic glyoxylate cycle has been previously hypothesized to occur in the peroxisomal compartment, which in the yeast *Saccharomyces cerevisiae* additionally represents the sole site for fatty acid β-oxidation. The subcellular location of the key glyoxylate-cycle enzyme malate synthase 1 (Mls1p), an SKL-terminated protein, was examined in yeast cells grown on different carbon sources. Immunoelectron microscopy in combination with cell fractionation showed that Mls1p was abundant in the peroxisomes of cells grown on oleic acid, whereas in ethanol-grown cells Mls1p was primarily cytosolic. This was reinforced using a green fluorescent protein (GFP)–Mls1p reporter, which entered peroxisomes solely in cells grown under oleic acid-medium conditions. Although growth of cells devoid of Mls1p on ethanol or acetate could be fully restored using a cytosolic Mls1p devoid of SKL, this construct could only partially alleviate the requirement for native Mls1p in cells grown on oleic acid. The combined results indicated that Mls1p remained in the cytosol of cells grown on ethanol, and that targeting of Mls1p to the peroxisomes was advantageous to cells grown on oleic acid as a sole carbon source.

Keywords: *Saccharomyces cerevisiae*; glyoxylate cycle; peroxisome; malate synthase 1; oleic acid.

Microorganisms are able to grow on nonfermentable carbon sources such as acetate, ethanol, or fatty acids, because they possess a glyoxylate cycle for generating four-carbon units that are suitable for biosyntheses of macromolecules. Similarly, plant seedlings can also use stored lipids as a sole carbon and energy source, by converting the acetyl-CoA product of fatty acid β-oxidation to four-carbon units using a cognate process. In those eukaryotes known to possess a glyoxylate cycle, e.g. plant seedlings and fungi, the process is thought to occur in the peroxisomal matrix.

Peroxisomes typically contain enzymes for reactions involving molecular oxygen and for metabolizing hydrogen peroxide [1]. This subcellular compartment represents the site of fatty acid β-oxidation, which in mammals is augmented by an additional process found in the mitochondria [2]. The significance of the fungal glyoxylate cycle to human health is underscored by the requirement of isocitrate lyase for the virulence of the pathogenic yeast *Candida albicans* [3]. Like the situation with *C. albicans*, *Saccharomyces cerevisiae* cells isolated from phagolysosomes obtained from infected mammalian cells similarly up-regulate isocitrate lyase as well as malate synthase, both of which represent key enzymes unique to the glyoxylate cycle [3]. As *S. cerevisiae* is a genetically more tractable yeast than *C. albicans*, it was chosen as a model fungal system for studying the glyoxylate cycle by analysing the subcellular distribution of malate synthase 1.

The *S. cerevisiae* glyoxylate cycle (Scheme 1) consists of five enzymatic activities, some of which are represented by isoenzymes: isocitrate lyase, Icl1p [4]; malate synthase, Mls1p and Dal7p [5]; malate dehydrogenase, Mdh1p [6], Mdh2p [7] and Mdh3p [8,9]; citrate synthase, Cit1p [10], Cit2p [11,12] and Cit3p/*YPR001w* [13]; and aconitase, Aco1p [14] and Aco2p/*YJL200c* [13]. As mentioned above, isocitrate lyase and malate synthase represent key enzyme activities that are unique to the glyoxylate cycle, whereas some of the remaining enzymes, e.g. mitochondrial Cit1p, Mdh1p, and Aco1p, are shared with the citric acid cycle. Icl1p is an extraperoxisomal protein, while Mdh3p and Cit2p are peroxisomal ones. The latter two enzymes end with a C-terminal SKL tripeptide representing a peroxisomal targeting signal PTS1 [15–17].

The two malate synthases Mls1p and Dal7p are also SKL-terminating proteins that are 81% identical to one another. However, as the *MLS1* gene is highly transcribed on nonfermentable carbon sources and is essential for cell growth on these media, whereas *DAL7* is not [5], it is reasoned that only Mls1p represents the malate synthase activity specifically involved in the glyoxylate cycle. Dal7p, whose peroxisomal location remains putative, is actually thought to be involved in the metabolism of glyoxylate produced during the degradation of allantoic acid to urea [5].

Initial work on peroxisomal citrate synthase (Cit2p) led to the conclusion that the glyoxylate cycle is a peroxisomal process [12]. However, the cycle's subcellular location is no longer clear because peroxisomal Cit2p has since been shown to be dispensable for the glyoxylate cycle [9] and, moreover, cells lacking peroxisomal malate dehydrogenase

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Abbreviations: PTS1, peroxisomal targeting signal type 1; YP, yeast extract/peptone; GFP, green fluorescent protein; Mls1p, malate synthase 1; Cit2p, peroxisomal citrate synthase.

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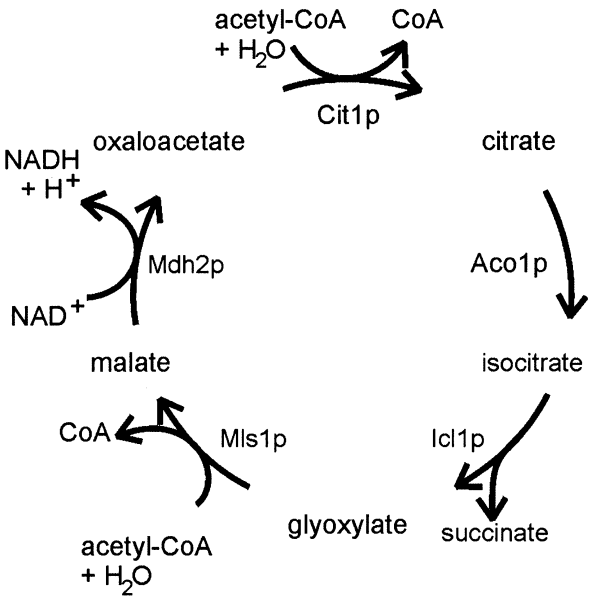


Scheme 1. The glyoxylate cycle in yeast cells grown on ethanol. To synthesize sugars from C_2 carbon sources, yeast cells rely on the glyoxylate cycle. This process is based on some of the same enzymes as those of the citric acid cycle. However, the steps in which decarboxylations occur in the latter cycle are bypassed using two glyoxylate-cycle specific enzymes, isocitrate lyase and malate synthase. The *S. cerevisiae* enzymes Icl1p, Mls1p, Mdh2p, Cit1p, and Aco1p are noted, these being essential for growth of yeast cells on C_2 carbon sources such as ethanol or acetate.

(Mdh3p) grow abundantly on ethanol [18]. Instead, the malate dehydrogenase activity specifically involved in the glyoxylate cycle is attributed to the cytosolic isoform Mdh2p [7]. The suggestion of an extra-peroxisomal location for the yeast glyoxylate cycle was further reinforced by the demonstration that Icl1p is a cytosolic enzyme [4], and that *pex* mutants lacking functional peroxisomes grow plentifully on ethanol as sole carbon source [19]. The present work was aimed at determining the subcellular location of the glyoxylate cycle by examining the partitioning of Mls1p in cells grown on media supplemented with ethanol or oleic acid.

MATERIALS AND METHODS

Strains, plasmid constructions and gene disruptions

S. cerevisiae strains, plasmids and oligonucleotides used are listed in Table 1. *Escherichia coli* strain HB101 was used for all plasmid amplifications and isolations. Construction of strains JD1, JR85, and JR86 has been described [5]. To remove the three codons for SKL from the *MLS1* gene, single-strand mutagenesis was performed according to the manufacturer's protocol (Amersham Pharmacia Biotech., Stockholm, Sweden) using oligonucleotide H161 (Table 1). To reintroduce the native *MLS1* or an *MLS1* variant lacking the SKL codons back to the genomic *MLS1* locus, strain JR86 was transformed with *URA3*-marked integrative plasmids pB10-WT or pB10-WT ΔSKL digested with *Pvu*II. These pUC18-based plasmids consisted of the promoter and terminator regions of *MLS1* delineating the open reading frame, with or without the codons for SKL, and *URA3* (Scheme 2). Integration of the disruption fragments resulted in the respective strains KM10 and KM11. Correct integration of these plasmid fragments was verified by polymerase chain reaction using oligonucleotide pairs H338 and H162, or H339 and H161, respectively (Table 1, Scheme 2).

To generate null mutants devoid of Mls1p, the corresponding gene was deleted by transforming strains BJ1991 [20] with an *mls1*Δ::*LEU2* disruption fragment [5]. Cells that had returned to leucine prototrophy were verified for growth deficiency on ethanol and acetate media and were designated strain KM12. The mutant phenotype was confirmed by complementation using native *MLS1* carried on a YEp352 multicopy vector, YEp352-MLS1 [5]. The BJ1991-derived strain KM13 expressing the SKL-less Mls1p was constructed and verified as described above for strain KM11. YEp352-MLS1ΔSKL was constructed by inserting a 2.3-kb *Sal*I fragment containing the complete *MLS1* gene into this multicopy vector, and replacing parts of the coding region with the single-strand mutagenized sequence, resulting in the expression of an SKL-truncated Mls1p (Mls1pΔSKL). The plasmid was introduced to strain JR86, resulting in strain KM15.

To create a reporter construct based on GFP extended by the C-terminal half of Mls1p comprising 274 amino acids of a total of 554, PCR was applied to YEp352-MLS1 template DNA using oligonucleotides H623 and H625 and *Pfu* high-fidelity polymerase (Stratagene, La Jolla, CA, USA). The single amplification product obtained was digested with *Sph*I and *Bgl*II, and ligated to an *Sph*I- and *Bam*HI-digested plasmid pJR233M [21], resulting in plasmid pLW89. Construction of the parent plasmid pJR233 is described elsewhere [22]. Nucleic acid manipulations [23] and yeast transformations [24] were performed as described.

Media and growth conditions

Plates contained 0.67% (w/v) yeast nitrogen base without amino acids (Difco), 3% (w/v) agar, amino acids as required, and either 2% (w/v) D-glucose, 2.5% (v/v) ethanol, or 0.1 M potassium acetate at pH 6.0. Fatty acid plates contained 0.125% (w/v) oleic acid, and 0.5% (w/v) Tween 80 to emulsify the fatty acids [25], but lacked yeast extract. For oleic acid utilization assays and cell fractionations, cells were grown overnight in rich-glucose medium consisting of YP (1% w/v yeast extract, 2% w/v peptone) and 2% D-glucose, transferred to YP containing 0.5% D-glucose at a 1 : 100 dilution, and grown to late log phase. Cells were transferred to water at a concentration of 10^4 cells·mL^{-1}, serially diluted (1 : 10 dilutions), and culture aliquots of 2.5 μL were applied to solid media [25,26].

Growth assays in liquid oleic acid medium were performed following a modified protocol [25,26]. Cells were grown overnight in synthetic medium (0.67% yeast nitrogen base with amino acids added) containing 2% D-glucose, and the cultures diluted to an D_{600} of 0.5 in synthetic medium containing 0.5% D-glucose and grown further with shaking at 30 °C. Upon reaching an D_{600} of 3.0 culture aliquots were removed and diluted to an D_{600} of 0.02 in synthetic media containing 0.03 M potassium phosphate buffer (pH 6.0), 0.1% yeast extract, and either 2% ethanol or 0.2% oleic acid and 0.02% Tween 80 (the latter carbon source adjusted prior

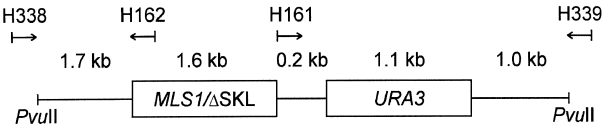
Table 1. *S. cerevisiae* strains, plasmids, and oligonucleotides used. The numbers in superscript following the strains' designation refer to their parental genotypes, e.g. JD[1] was derived from (1) GA1-8C.

Strain, plasmid, or oligonucleotide	Description	Source or Reference
Strains		
(1) GA1-8C	*MAT***a** *ura3-52 leu2 his3 trp1-1 ctt1-1 gal2*	[5]
JD1[1]	*dal7*Δ::*HIS3*	[5]
(2) JR85[1]	*mls1*Δ::*LEU2*	[5]
(3) JR86[2]	*mls1*Δ::*LEU2 dal7*Δ::*HIS3*	[5]
KM10[3]	*URA3*, expressing Mls1pΔSKL from the *MLS1* locus	This study
KM11[3]	*URA3*, expressing Mls1p from the *MLS1* locus	This study
(4) BJ1991	*MAT*α *leu2 ura3-52 trp1 pep4-3 prb1-1122 gal2*	[20]
(5) KM12[4]	*mls1*Δ::*LEU2*	This study
KM13[5]	Expressing Mls1pΔSKL from the *MLS1* locus	This study
KM15[3]	Over-expressing Mls1pΔSKL from a multicopy vector	This study
Plasmids		
pB10-WT	Plasmid for reintroducing *MLS1* at the native locus	This study
pB10-WTΔSKL	As above, for introducing an *MLS1* truncation	This study
YEp352-MLS1	Multicopy vector harboring native *MLS1*	[5]
YEp352-MLS1ΔSKL	Multicopy vector harboring a truncated *MLS1*	This study
pJR233	YEp352-based plasmid expressing GFP-SKL	[22]
pJR233M	pJR233-derived vector for GFP fusions	[21]
pLW89	pJR233M-derived plasmid expressing GFP-Mls1p	This study
Oligonucleotides		
H161	5′-CACTGATTTGTGAGAATTCTGATCTCC-3′	This study
H162	5′-CAATGAACTCTAGAGC-3′	This study
H338	5′-GATACTAAGTGAGCTTAAGGAGG-3′	This study
H339	5′-CCCGACGCCGGACGAGCCCGC-3′	This study
H623	5′-AGAAAGATCTATCTAGTGGGTTGAATTGCGGACGTTGG-3′	This study
H625	5′-AGAAGCATGCGATCACAATTTGCTCAAATCAGTGGGCGTCGCC-3′	This study

to dilution to pH 7.0 with NaOH). The D_{600} of the cultures was determined at the times indicated. For vital counts, culture aliquots were removed following the indicated periods and plated on solid YP medium containing 2% D-glucose for enumeration following 2 days incubation.

Cell fractionation and immunoblotting

Late log-phase cells were harvested by centrifugation and transferred to YP medium containing 2.5% ethanol, or 0.2% oleic acid and 0.02% Tween 80 (pH adjusted as mentioned above). Following growth for at least 9 h at 30 °C with shaking, cells were harvested by centrifugation (5000 ***g***), and total homogenates, organellar pellets, and postorganellar supernatants were prepared as described [27]. A 10% portion of each of the fractions (postnuclear supernatant, organellar pellet or cytosolic supernatant) was used for protein precipitation. These organellar or supernatant fractions were made up to 0.5 mL with breaking buffer [27], followed by 5 μL Triton X-100 (final concentration 1% v/v) and an appropriate amount of 80% (w/v) trichloroacetic acid to obtain a 10% final concentration of trichloroacetic acid. The resulting oily pellet was washed once with a diethyl ether/ethanol mixture (1 : 1), which removed traces of Triton X-100 and trichloroacetic acid, and dissolved in 30 μL 0.1 M NaOH. To the solubilized protein a volume of 30 μL sample buffer (100 mM Tris/HCl at pH 6.7; 20% w/v glycerol; 2.0% w/v SDS; 6 M urea; 100 mM dithiothreitol; and 0.1% w/v bromophenol blue) was added, and the mixture was heated to 80 °C prior to resolution by electrophoresis on an SDS/polyacrylamide gel (10% w/v) [28]. Following electrophoresis, the resolved proteins were transferred to a nitrocellulose filter according to a standard protocol. Detection of the immobilized proteins was performed by adding a primary antibody against Mls1p (diluted 1 : 2000) or peroxisomal catalase A (Cta1p, diluted 1 : 1000) [27], followed by application of the enhanced chemiluminescence (ECL) system from Pierce (Super Signal West Pico Chemiluminiscent Substrate; no. 34083). Determination of protein concentration was performed as described [29].



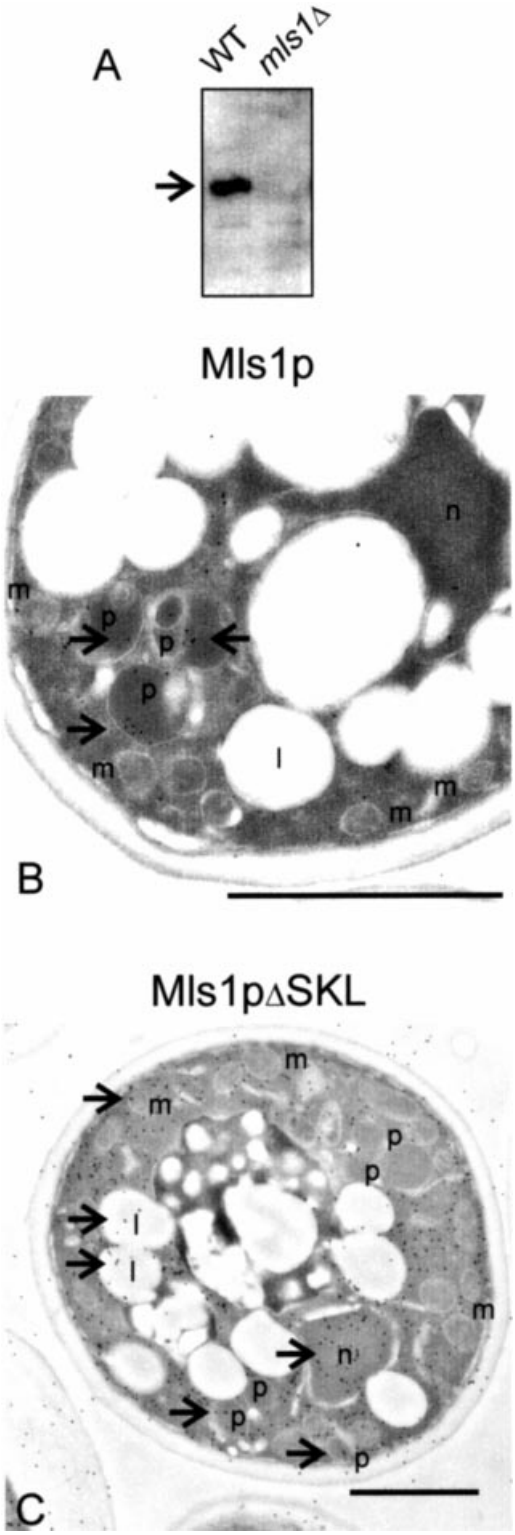
Scheme 2. Diagram of plasmid construction. The pB10-WT or pB10-WTΔSKL constructs for expressing Mls1p or Mls1pΔSKL from the native locus are shown. Not to scale. PCR oligonucleotide H338 primes 0.25 kb 5′ of the *Pvu*II site, H162 primes 0.1 kb 3′ of the *MLS1* ATG start site, H161 primes at a site that includes the *MLS1* stop codon, and H339 primes 0.3 kb 3′ of the *Pvu*II site.

Purification of tagged Mls1p and generation of anti-Mls1p Ig

To obtain pure protein for generating an antibody against Mls1p, the pQE-32 expression system (Qiagen Inc., Valencia, CA, USA) was used. A DNA fragment encoding the

C-terminal 308 amino acids (out of a total of 554) was used to express a soluble His-tagged protein (His_6-Mls1p) in bacterial cells. Cell lysates were subjected to affinity chromatography using a Ni^{2+}-containing Sepharose 6B column (Pharmacia), and protein was purified to near homogeneity using a Ni-nitrilotriacetic acid Spin Kit (Qiagen). SDS/PAGE revealed a protein band with an apparent molecular mass of 38 000, which corresponded to the deduced size of the His_6-Mls1p truncation (not shown).

A fraction of a purified His_6-Mls1p was immobilized on a membrane and subjected to tryptic digestion, and HPLC-purified peptide fragments were microsequenced. The sequences obtained, GVHAMGGMAAQIPIK and ATPTDLSK, corresponded to the respective deduced residues 334–348 and 546–553 of Mls1p, confirming the identity of the purified recombinant protein. The same purified protein (100 μg) in combination with complete Freund's adjuvant (3 mL total volume) was used to immunize rabbits (approved by the Ethics Committee of the University of Vienna). This was followed by three additional booster injections. After ammonium sulfate precipitation and DEAE-ion exchange of the antiserum, antibody was used for immunoblotting. For immunoelectron microscopy, the antibody preparation was subjected to affinity purification using membrane-immobilized soluble protein extracts obtained from yeast cells over-expressing native Mls1p.



RESULTS

The subcellular location of Mls1p

Malate synthase 1 terminates with an SKL tripeptide representing a peroxisomal targeting signal PTS1 [5,15]. To determine whether Mls1p is indeed a peroxisomal protein, electron microscopy was performed using an anti-Mls1p antibody that was generated against a recombinant protein comprising the C-terminal 308 amino acids of Mls1p. Although it cannot be entirely ruled out that the antibody used additionally cross-reacts with Dal7p, which is 81% identical to Mls1p and also ends with SKL, expression of Dal7p in cells grown in the presence of ample nitrogen was considered to be unlikely as transcription of the corresponding *DAL7* gene is tightly repressed under these medium conditions [5].

Purified antibody was applied to a filter containing soluble protein extracts obtained from wild-type and *mls1*Δ cells that were propagated in rich medium supplemented with ethanol. This resulted in a protein band with a molecular mass of 62 000 in the lane with the wild-type extract that was absent from the lane corresponding to the *mls1*Δ mutant (arrow; Fig. 1A), thereby confirming the specificity of the antibody. Application of the antibody to thin sections of wild-type cells grown on oleic acid medium

Fig. 1. SKL is required to direct Mls1p to the peroxisomes under oleic acid-medium conditions. (A) Specificity of the anti-Mls1p antibody. Extracts from homogenized wild-type (GA1-8C) and *mls1*Δ yeast (JR85) strains were immobilized on a membrane to which anti-Mls1p Ig was applied. A single protein band with a molecular mass of 62 000 is seen only in the lane representing the wild-type extract (arrow). (B) Immunoelectron micrograph of a wild-type yeast cell expressing native Mls1p from the chromosomal locus (GA1-8C). Gold particles representing Mls1p in the matrix of peroxisomes are indicated (arrows). l, lipoidal inclusion; m, mitochondrion; n, nucleus; and p, peroxisome. The bar is 1 μm. (C) Micrograph of an *mls1*Δ mutant over-expressing an SKL-less Mls1p (KM15). Gold particles (marked with arrows) are seen in the nucleus, cytoplasm, and in some case also in mitochondria, peroxisomes, and lipoidal inclusions. The bar and letters are equivalent to those in (B).

resulted in the decoration of peroxisomes (Fig. 1B). This result lent credence to the suggested peroxisomal location of Mls1p based on a GFP-Mls1p green fluorescent protein reporter expressed in cells grown on oleic acid [30]. Use of this antibody with thin sections of an otherwise isogenic *mls1*Δ*dal7*Δ strain over-expressing an SKL-less Mls1p variant (Mls1pΔSKL; strain KM15) on oleic acid revealed gold particles decorating both the nucleus and cytosol (Fig. 1C), which was consistent with a noncompartmentalized antigen. The results indicated that the SKL tripeptide was important for peroxisomal targeting.

Peroxisomal import of Mls1p depends on oleic acid

The glyoxylate cycle is essential for cell growth on media supplemented with nonfermentable carbon sources not requiring peroxisomes for their metabolism, e.g. ethanol or acetate, and is physiologically functional in mutant *pex* cells lacking a normal peroxisomal compartment [19]. This raised the issue of whether Mls1p is compartmentalized during growth of cells under such medium conditions. To examine the subcellular location of malate synthase 1 in cells grown on ethanol, a GFP reporter was constructed that was extended with the C-terminal 274 amino acids of Mls1p (out of a total of 554), including the terminal SKL. Expression of this GFP-Mls1p was compared to that of a control GFP extended solely by SKL (GFP-SKL). GFP-SKL has been amply shown before to be imported into the peroxisomes of wild-type cells, but to remain cytosolic in *pex* mutant cells devoid of functional peroxisomes [22,31]. The results demonstrated that living yeast cells expressing either GFP-Mls1p or GFP-SKL on oleic acid exhibited bright, closely bunched fluorescent points (Fig. 2, upper panels). On the other hand, in cells grown on ethanol, the punctate pattern of fluorescence due to GFP-SKL was less dense, whereas fluorescence due to GFP-Mls1p was altogether diffuse (Fig. 2, lower panels). This indicated that unlike the situation with GFP-SKL, which was targeted to peroxisomes in cells grown under both medium conditions, compartmentalization of GFP-Mls1p into peroxisomes depended on cell growth on oleic acid medium.

To reinforce the evidence for the differential subcellular location of Mls1p, cellular fractionation was used. Fractions were prepared from ethanol-grown cells that contained import-competent peroxisomes as they could compartmentalize GFP-SKL efficiently (Fig. 2). Lysates of homogenized wild-type cells were spun to yield an organellar pellet consisting of mitochondria and peroxisomes, and a cytosolic supernatant. Equal fractions of each of the protein preparations (10% of total vol) were immobilized on replicate membranes to which were applied antibodies against Mls1p or yeast peroxisomal Cta1p. The results demonstrated that although Mls1p was clearly detectable in both the total homogenate and the supernatant (lanes 1 and 2 in the upper panel; Fig. 3A), in the peroxisome-enriched organellar pellet levels of Mls1p were below the detection limit (lane 3; Fig. 3A). Cta1p was visible in all three lanes, but was especially abundant in the pellet (lane 3 in the lower panel; Fig. 3A). Hence, during cell growth under ethanol medium conditions, peroxisomal Cta1p was imported, but not Mls1p.

Fractionation was also performed on oleic acid-grown cells expressing native Mls1p or Mls1pΔSKL (designated in Fig. 3B as + or – SKL, respectively). Under these conditions, both Mls1p and Cta1p were found in the organellar pellet from cells expressing native Mls1p (lane 5; Fig. 3B). A fairly high proportion of Mls1p and Cta1p was seen in both the supernatant and pellet fractions; it is not yet possible to isolate completely 100% intact organelles. On the other hand, Mls1pΔSKL- which could be detected in the homogenate and supernatant (lanes 2 and 4) was absent from the corresponding organellar pellet (lane 6). These results confirmed the requirement of SKL for peroxisomal import, and reiterated that the compartmentalization of malate synthase 1 depended on cell growth on oleic acid medium.

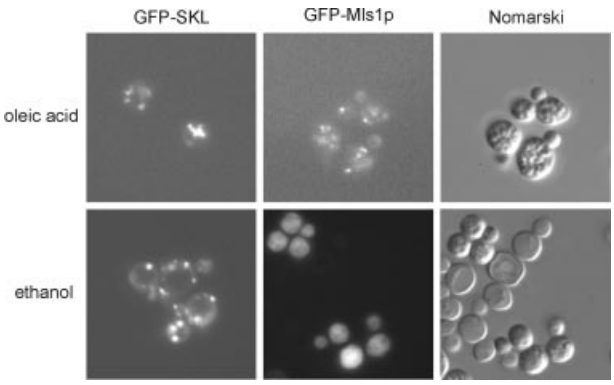


Fig. 2. Subcellular localization of GFP-Mls1p. Oleic acid-grown BJ1991 cells transformed with GFP-Mls1p or GFP-SKL were monitored by direct fluorescence microscopy. Punctate fluorescence indicated presence of GFP in peroxisomes. The diffuse fluorescence seen in ethanol-grown cells expressing GFP-Mls1p was commensurate with a cytosolic localization of the reporter protein. Nomarski images corroborated the integrity of the cells examined.

Targeting of Mls1p to peroxisomes is advantageous for growth on oleic acid

Two steps of the glyoxylate cycle take place in the cytosol: the splitting of isocitrate into succinate and glyoxylate, and the dehydrogenation of malate to oxaloacetate (Scheme 1).

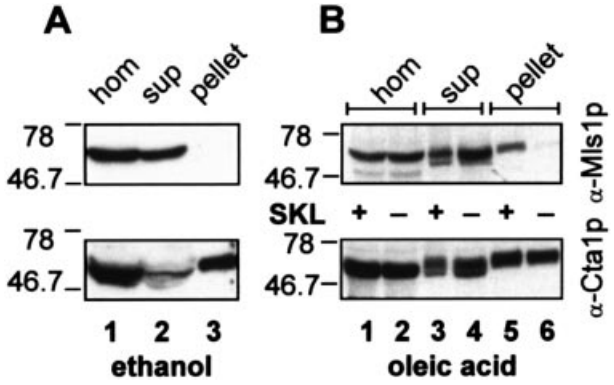


Fig. 3. Subcellular distribution of native Mls1p under oleic acid- and ethanol medium conditions. (A) Ethanol-grown KM11 cells or (B) oleic acid-grown KM11 and KM10 cells (+ or –SKL, respectively) were used for cell fractionation. Aliquots representing 10% of each volume from the primary homogenate (hom), the organellar pellet (pellet), or supernatant (sup) were immobilized to duplicate membranes which were probed with anti-malate synthase (α-Mls1p) or anti-catalase A (α-Cta1p) Ig. Molecular mass markers (kDa) are indicated to the left.

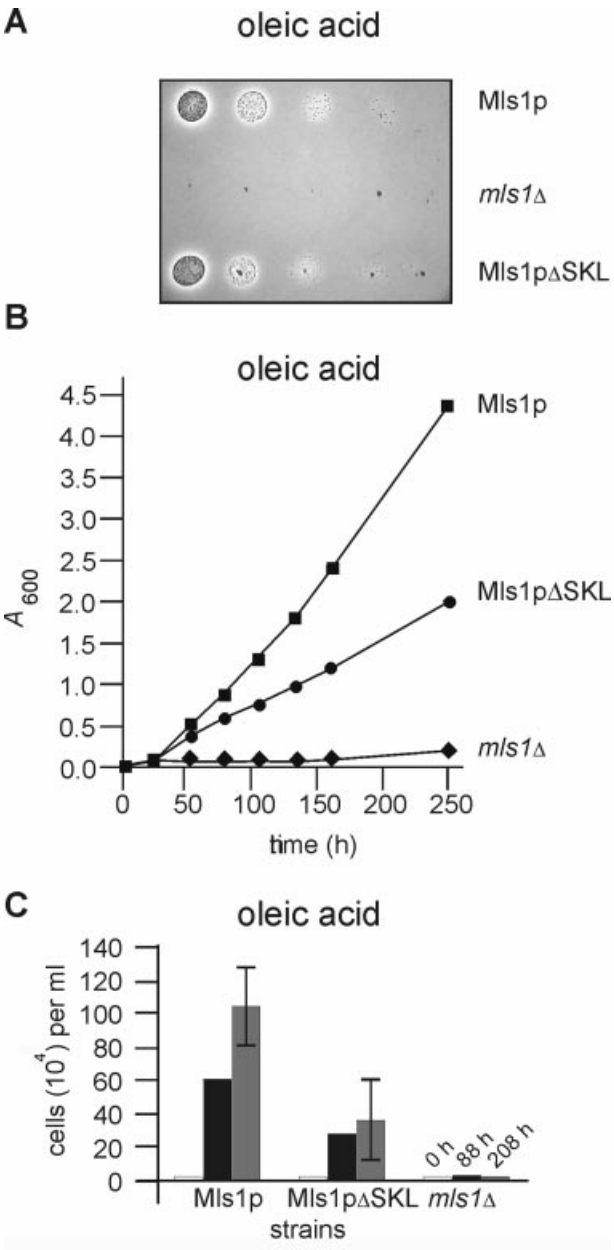


Fig. 4. Growth of cells on oleic acid. (A) Plate assay for the utilization of oleic acid. Yeast *mls1*Δ cells expressing Mls1p in its native form or without SKL were compared with an otherwise isogenic null mutant for formation of clear zones in oleic acid medium lacking yeast extract. Strains were grown to late log-phase in rich-glucose medium, and serially diluted culture aliquots were applied to the plates. The plate was recorded photographically following 5 days incubation at 30 °C. The strains used were BJ1991 (wild type), KM12, and KM13. (B) Cell growth in liquid medium. The strains used were wild type cells (BJ1991, ■), *mls1*Δ cells (KM12, ◆), or *mls1*Δ cells complemented with Mls1pΔSKL (KM13, ●). The curves represent the average of three independent experiments. (C) Vital counts of diluted culture aliquots from (B) that were plated on YPD medium. Bars represent standard error ($n = 3$).

However, the intervening activity undertaken by Mls1p, i.e. formation of malate from glyoxylate and acetyl-CoA, occurs in the peroxisomes when cells are grown on oleic acid. This prompted the question of whether there is any advantage to cells targeting Mls1p to peroxisomes, as by doing so cells partition the enzyme reactions to either side of the organellar membrane. To examine the requirement for compartmentalizing Mls1p, yeast *mls1*Δ cells (KM12) and strains expressing native Mls1p or Mls1pΔSKL from the chromosomal locus (strains KM13 and KM15) were grown on solid fatty acid medium. The medium used also contained Tween 80, which acted to disperse the fatty acids but was also a poor carbon source. Hence, mutant cells often grow to some extent on these plates but transparent zones in the opaque medium around regions of cell growth indicate utilization of the fatty acid substrate [25]. Application of serial dilutions of cell cultures (BJ1991, KM12, KM13) to this medium showed that the *mls1*Δ mutant was unable to form a clear zone (Fig. 4A). On the other hand, despite representing a strictly cytosolic protein, Mls1pΔSKL appeared to overcome the mutant phenotype (Fig. 4A).

To examine whether a cytosolic malate synthase was as efficient as a peroxisomal one for maintaining a functional glyoxylate cycle on oleic acid, liquid growth assays were conducted. The results showed that the growth rate of cells expressing wild-type Mls1p was higher compared with those producing Mls1pΔSKL (Fig. 4B). Vital counts based on this assay served to confirm that although the compartmentalization of malate synthase was not strictly essential, it was advantageous for cells to grow on oleic acid (Fig. 4C). The greater sensitivity of liquid growth assays on oleic acid compared with solid medium has been previously reported [32].

As a control, cells were streaked on ethanol, acetate, or glucose media (Fig. 5A). The results demonstrated that the *mls1*Δ mutant failed to grow on ethanol or acetate. However, expression of either of the two Mls1p constructs complemented the *mls1*Δ mutant phenotype on these media. Growth assays in liquid medium supplemented with ethanol similarly showed that although *mls1*Δ cells were unable to multiply, those cells expressing malate synthase in any form, i.e. Mls1p or Mls1pΔSKL, grew abundantly (Fig. 5B). This indicated that a constitutively cytosolic Mls1p was sufficient for cells to maintain the metabolite flux through the glyoxylate cycle during growth on nonfermentable carbon sources other than fatty acids.

DISCUSSION

The requirement for the compartmentalization of the yeast glyoxylate cycle into peroxisomes has been put into question in light of chronicled observations of growth of *S. cerevisiae pex* mutants devoid of functional peroxisomes on ethanol [19]. In addition, *pex* mutants have also been demonstrated to undergo normal meiosis and sporulation in liquid acetate medium [33], processes which similarly require a functional glyoxylate cycle [34]. However, as *pex* mutants fail to grow or sporulate in liquid oleic acid medium [33], the issue of the partitioning of the glyoxylate cycle in cells grown under fatty acid-medium conditions has hitherto remained open.

We showed here that one of the key glyoxylate-cycle enzymes, Mls1p, was cytosolic in cells grown on ethanol, whereas in cells grown on oleic acid Mls1p was peroxisomal. This is the first time that the targeting of an SKL-terminating protein into peroxisomes is shown to be different depending on the growth conditions. A previous study on the subcellular distribution of AKL-terminated

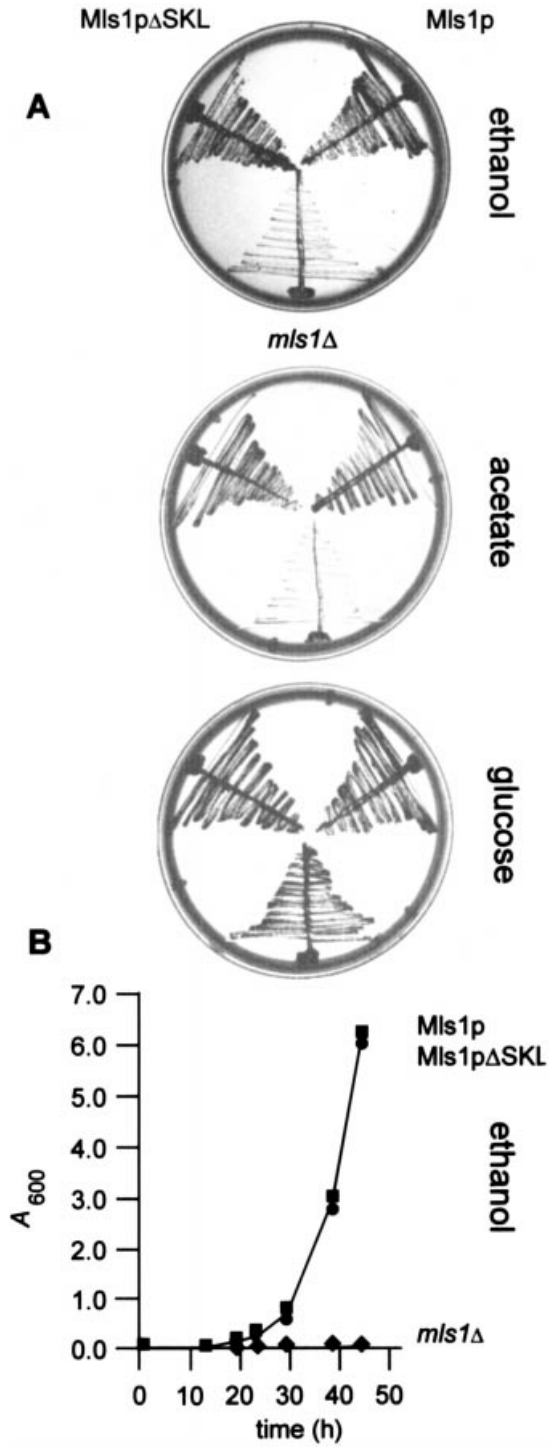


Fig. 5. Growth of cells on ethanol. (A) Plate assays for functional complementation of a yeast *mls1*Δ strain (JR86) expressing native Mls1p (KM11) or an SKL-less variant (KM10) on ethanol, acetate, or glucose media, as indicated. (B) Cell growth in liquid ethanol medium. The strains used were identical to those in Fig. 4. The curves represent the average of three independent experiments.

aspartate aminotransferase Aat2p demonstrated that this protein was compartmentalized in cells grown on oleic acid, but remained in the cytosol of glucose-grown cells [35]. However, under these latter conditions peroxisomes are very few due to catabolite repression [36,37], whereas on ethanol peroxisomes are not only more readily detectable, but are additionally import competent (Fig. 2). This means that unlike the situation with Aat2p which essentially has no target compartment in cells grown on glucose, Mls1p was selectively retained in the cytosol of cells propagated on ethanol. Interestingly, the C-termini of both Mls1p and Aat2p contain acidic amino-acid residues at the 5th-last position with respect to the terminal residue (DLSKL in Mls1p and EISKL in Aat2p), which is unusual at this position [21]. The significance of this similarity is currently being addressed.

Demonstration of the cytosolic location of Mls1p in wild-type cells grown on ethanol completes the picture of the extra-peroxisomal location of the glyoxylate cycle in yeast grown on carbon sources other than fatty acids. The only other key enzyme unique to the glyoxylate cycle, Icl1p, is also extra-peroxisomal [4], as are the other enzymes essential for the glyoxylate cycle (Scheme 1) including mitochondrial citrate synthase encoded by *CIT1* (and possibly also by *CIT3*), cytosolic Mdh2p, and extra-peroxisomal Aco1p.

As mentioned previously, malate synthase catalyses the formation of malate from glyoxylate and acetyl-CoA, the source of the latter being either peroxisomal when breaking down fatty acids, or cytosolic when extra-cellular two-carbon substrates are used. Although not strictly essential, the peroxisomal localization of malate synthase 1 appears to be advantageous for cells growing on oleic acid, in that acetyl-CoA production and utilization are thereby intimately compartmentalized together to increase efficiency. Future work on the entry of glyoxylate into peroxisomes will help elucidate how the glyoxylate cycle proceeds across an organellar membrane in cells grown on oleic acid. In addition, solution of the crystal structure of Mls1p could also turn out to be helpful in elucidating whether the protein's selective import into peroxisomes might have something to do with the exposure of the C-terminal SKL tripeptide for making contact with the cognate receptor Pex5p.

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