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Yeast Functional Analysis Report

The ORF *YNL274c* (*GOR1*) codes for glyoxylate reductase in *Saccharomyces cerevisiae*

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Abstract

The enzyme glyoxylate reductase reversibly reduces glyoxylate to glycolate, or alternatively hydroxypyruvate to D-glycerate, using either NADPH or NADH as a co-factor. The enzyme has multiple metabolic roles in different organisms. In this paper we show that *GOR1* (ORF *YNL274c*) encodes a glyoxylate reductase and not a hydroxyisocaproate dehydrogenase in *Saccharomyces cerevisiae,* even though it also has minor activity on α-ketoisocaproate. In addition, we show that deletion of the glyoxylate reductase-encoding gene leads to higher biomass concentration after diauxic shift. Copyright © 2006 John Wiley & Sons, Ltd.

Keywords: ***Saccharomyces cerevisiae*; glyoxylate reductase; hydroxypyruvate; hydroxyisocaproate**

Introduction

The enzyme glyoxylate reductase catalyses the interconversion between glyoxylate and glycolate, or alternatively between hydroxypyruvate and D-glycerate, using either NADP(H) (EC 1.1.1.79) or NAD(H) (EC 1.1.1.26) as a co-factor. This enzyme is found in various organisms, including prokaryotes, eukaryotes and archaea. It is often present as more than one isoenzyme, and in eukaryotes these may be localized in different cell compartments (Givan and Kleczkowski, 1992; Nuñez *et al*., 2001).

Glyoxylate reductase has multiple metabolic roles. In methylotrophic bacteria it participates in serine biosynthesis (Chistoserdova and Lidstrom, 1992; Nuñez *et al*., 2001). In plants, this enzyme is suggested to have a role in photorespiration, during which process glyoxylate is generated as an intermediate (Givan and Kleczkowski, 1992; Kleczkowski *et al*., 1986). In addition, the reaction carried out by NADPH-linked glyoxylate reductases may act as a sink for excess NADPH formed in chloroplasts during photosynthesis (Tolbert *et al*., 1970). In humans, hydroxypyruvate/glyoxylate reductase is thought to play a key role in directing the carbon to gluconeogenesis by its ability to convert hydroxypyruvate to D-glycerate (Holmes and Assimos, 1998). The hereditary disease primary hyperoxaluria II is caused by a deficiency of this enzyme which leads to excretion of oxalate and L-glycerate in urine (Cramer *et al*., 1999; Williams and Smith, 1968).

In *Saccharomyces cerevisiae*, two glyoxylate reductase isoenzymes have been purified (Fukuda *et al*., 1980; Tochikura *et al*., 1979) but their physiological role is not known, neither have the corresponding genes been identified. However, a BLAST search of the *S. cerevisiae* database suggests seven ORFs as possibly coding for glyoxylate reductase. One of these is ORF *YNL274c*, which has been indicated, along with two other ORFs, *YER081w* and *YIL074c*, as a homologue of hydroxyisocaproate dehydrogenase (Dickinson *et al*., 1997). Hydroxyisocaproate dehydrogenase carries out the interconversion between α-ketoisocaproate

and D-2-hydroxyisocaproate in *Lactobacillus casei* (Lerch *et al.*, 1989). NADH-dependent hydroxyisocaproate dehydrogenase activity has been observed in ethanol-grown yeast cells, and appeared to occur within the mitochondrion. Furthermore, whereas the hydroxyisocaproate dehydrogenase activity reached its maximum in glucose-grown cells just before glucose was depleted, the expression of the ORF *YNL274c* was shown to be repressed by glucose (Albers *et al.*, 2003).

In the present study, we show that the ORF *YNL274c* does not code for a hydroxyisocaproate dehydrogenase but for a glyoxylate reductase of *S. cerevisiae,* although it does have some activity towards α-ketoisocaproate. We also show that deletion of the glyoxylate reductase-encoding gene leads to higher biomass concentration after diauxic shift.

Materials and methods

Strains and plasmids

Yeast deletion strains were obtained from EUROSCARF (**http://www.uni-frankfurt.de/fb15/mikro/euroscarf/col_index.html**; accessed 20 December 2005). The parent strain of the deletion strains was BY4742 (*MAT*α, *his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0*), which was derived from S288C (Brachmann *et al.*, 1998). Otherwise, *S. cerevisiae* CEN.PK2-1D (VW-1B; *MAT*α, *leu2-3/112 ura3-52 trp1-289 his3*Δ*1 MAL2-8*^{c} *SUC2*) (Boles *et al.*, 1996) was used as the host strain. *Escherichia coli* strain DH5α (Woodcock *et al.*, 1989) was used as the bacterial cloning host.

For the overexpression studies the ORFs *YNL274c, YER081W, YIL074c, YOR388c, YPL113c* and *YGL185c* were amplified by PCR from the genomic DNA of *S. cerevisiae* strain S288C. Primers used in the PCR amplification are given in Table 1. The PCR products were cloned to either the *Bam*HI site of pUC19 (Yanisch-Perron *et al.*, 1985) or to the TOPO-TA® vector (Invitrogen, USA) and sequenced with the same primers used in the PCR amplification reactions. In order to clone the ORF encoding *FDH1*, a longer fragment of the genomic DNA was cloned first to avoid obtaining the homologous *FDH2* sequence during the PCR amplification. This longer PCR product was subsequently used to clone the ORF for *FDH1*. The cloned ORFs were transferred to the *Bgl*II site of an expression cassette containing *PGK1* promoter and terminator (Mellor *et al.*, 1983), at the *Hind*III site of YEplac195 yeast vector (Gietz and Sugino, 1988). The coding regions were again sequenced. The expression plasmids were transformed to the CEN.PK2-1D yeast strain using the lithium acetate method (Gietz *et al.*, 1992; Hill *et al.*, 1991).

Cultivation conditions

Cultivations for enzyme activity studies of overexpression and deletion strains were carried out using the yeast synthetic complete medium (YSC) modified from (Sherman *et al.*, 1983), with the addition of glucose (20 g/l). Uracil was omitted as appropriate for plasmid selection. Pre-inocula

Table 1. Primers used to construct overexpression strains of the six putative glyoxylate reductase homologues in *S. cerevisiae*

Gene		Primer
YPL113c	frw	AGTCAGATCTATGATTACTTCAATTGACATAGCAG
	rev	AGTCAGATCTTCAGTTGAGCACATACTTACCATCAC
YOR388c	frw1	AGTCAGATCTGTTTGGCTATGGTTTAAAACATTC
	frw2	AGTCAGATCTATGTCGAAGGGAAAGGTTTTGCTGG
	rev	AGTCAGATCTTTATTTCTTCTGTCCATAAGCTCTGG
YNL274c	frw	AGTCAGATCTAATATGAGTAAGAAACCA
	rev	AGTCAGATCTTCAAACTAATGGCTTAGA
YIL074c	frw	AGTCAGATCTATGTCTTATTCAGCTGCCGATAATT
	rev	AGTCAGATCTTTAGTATAATAACCTGATGGAAACT
YGL185c	frw	ACGCGGATCCATGTGCGATTCTCCTGCAACGACTGG
	rev	ACGCGGATCCTCAAACTACACGGGAGAAATGCTCATC
YER081w	frw	ACGCGGATCCATGACAAGCATTGACATTAACAAC
	rev	ACGCGGATCCTTAATATAGCAATCTAATTGAGATC

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were grown from a single colony in 5 ml medium at 30 °C overnight, with 250 r.p.m. shaking. The 5 ml preinocula were transferred to 45 ml of fresh medium in 250 ml Erlenmeyer flasks and incubated for 24 h at 30 °C, 250 r.p.m. before the cells were harvested. Cultivations for the growth experiments were carried out using the same medium as above, except that the pH was adjusted to 5.0 with 50 mM phthalic acid. Pre-inocula were grown from several colonies in 10 ml medium at 30 °C overnight, with 250 r.p.m. shaking. The cultivations were carried out in 50 ml medium in 250 ml Erlenmeyer flasks, starting from $OD_{600} = 0.01$.

Enzyme activity assays

Enzyme activities were measured as nanokatals (nkat) per mg of total soluble protein. One nkat is defined by one nM NAD(P) formed per second. Enzyme activities were measured from cell extracts prepared by disrupting the yeast cells with glass beads in 50 mM Hepes buffer, pH 7.0, supplemented with the protease inhibitors PMSF and Pepstatin A (final concentration 0.17 and 0.01 mg/ml, respectively). The protein concentration of the cell extracts was determined with the Bio-Rad Protein Assay (Bio-Rad Laboratories, USA), using bovine serum albumin as a standard. All enzyme activities were measured in duplicate, using the Cobas Mira automated analyser (Roche, Switzerland) at 30 °C. The assay mixture for the activity measurement of glyoxylate reductase was 0.2 mM for NADPH in 50 mM sodium phosphate buffer. In some assays NADPH was replaced with equimolar NADH to test the NADH-specificity. The reaction was started by the addition of either sodium glyoxylate or lithium hydroxypyruvate to a final concentration of 25 mM. The hydroxyisocaproate dehydrogenase activity was measured according to Albers *et al.* (2003), using either NADPH or NADH as co-factor.

Results

The glyoxylate reductase homologues of *S. cerevisiae*

Glyoxylate reductases have to date been purified from many organisms, but the sequence of the encoding gene is known from only a few. At the start of this work, three sequences were found in the protein databanks, glyoxylate reductases of human, *Hyphomicrobium methylovorum* and *Methylobacterium extorquens.* We used these known sequences as query sequences for a BLAST search against the genome of *S. cerevisiae*. The best homologues were the following seven ORFs: (a) *YNL274c*, which had been assigned as a potential hydroxyisocaproate dehydrogenase (Dickinson *et al.*, 1997); (b) *YER081w* (*SER3*) and (c) *YIL074c* (*SER33*), identified as phosphoglycerate dehydrogenases (Albers *et al.*, 2003); (d) *YOR388c*, shown to be a formate dehydrogenase, *FDH1* (Overkamp *et al.*, 2002) and (e) *YPL275w/YPL276w*, which together are highly homologous to *YOR388c*, but are separated by a stop codon in the genomic reference strain S288C, but not in some other strains, and thus have been denoted as *FDH2* (Overkamp *et al.*, 2002); (f) *YPL113c* and (g) *YGL185c*, which have similarity to hydroxyacid dehydrogenases. All these ORFs, except the two ORFs encoding *FDH2* with a stop codon in the S288C strain (*YPL275w/YPL276w)* were investigated further.

A deletion strain of ORF *YNL274c* shows decreased glyoxylate reductase activity

Deletion strains of all the six ORFs, except *YOR388c* (encoding *FDH1*) were available from EUROSCARF. The deletion strains were tested for glyoxylate reductase activity (Figure 1). The glyoxylate reductase activity of the mother strain BY4742 was 0.6 ± 0.1 nkat/mg protein. The activities in the deletion strains of ORFs *YER081w, YIL074c, YPL113c* and *YGL185c* were similar; 0.6 ± 0.2–0.9 ± 0.4 nkat/mg protein. However, in the deletion strain of ORF *YNL274c*, the activity, 0.2 ± 0.1 nkat/mg protein, was only one-third of the activity in the mother strain.

Overexpression strains of ORFs *YNL274c, YGL185c* and *YPL113c* show elevated glyoxylate reductase activity

Since the presence of glyoxylate reductase activity in the deletion strains did not exclude the possibility that *YPL113c, YIL074c, YGL185c* or *YER081w*, in addition to *YNL274c*, may also encode a protein having glyoxylate reductase activity in *S. cerevisiae*, overexpression strains of the six ORFs were constructed. Two yeast transformants overexpressing each ORF were assayed for glyoxylate reductase activity (Table 2). Two known

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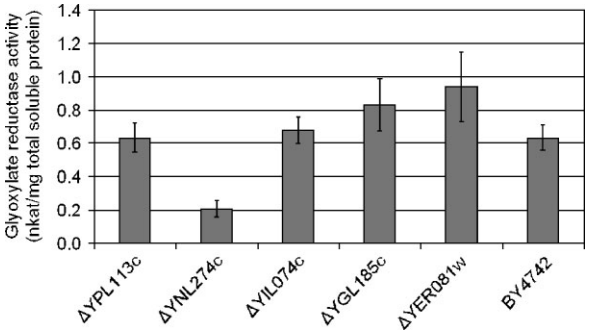

Figure 1. Glyoxylate reductase activity in the deletion strains of ORFs *YGL185c, YPL113c, YNL274c, YER081w* and *YIL074c*, and their parent strain BY4742 grown on YSC medium with 20 g/l glucose for 24 h. The activities were measured as duplicates from three independent cultivations. Error bars represent ± SEM

substrates, glyoxylate and hydroxypyruvate, of the baker's yeast glyoxylate reductases (Fukuda *et al*., 1980; Tochikura *et al*., 1979) were used. The activity on both glyoxylate and hydroxypyruvate of the CEN.PK2-1D control strain with the empty expression vector was below 1.0 nkat/mg protein.

Overexpression of ORFs *YOR388c, YER081*w and *YIL074c*, encoding formate dehydrogenase *FDH1*, and phophoglycerate dehydrogenases *SER3* and *SER33*, respectively, did not increase glyoxylate reductase activity against either substrate, compared to the control strain. In contrast, multicopy expression of the two ORFs *YGL185c* and *YPL113c*, encoding proteins with similarity to hydroxyacid dehydrogenases, resulted in a four- to ten-fold increase in the activity towards both substrates. The activity of these enzymes towards glyoxylate was two-fold compared to the activity on hydroxypyruvate. This ratio is similar to that measured for the isoenzyme glyoxylate reductase II of *S. cerevisiae* (Fukuda *et al*., 1980; Tochikura *et al*., 1979).

Table 2. Glyoxylate reductase activities, nkat/mg total soluble protein, in CEN.PK2-1D *S. cerevisiae* overexpressing yeast ORFs with homology to known glyoxylate reductases

	glyoxylate	hydroxypyruvate
YPL113c	2.16 ± 0.86	1.33 ± 1.06
YOR388c	0.63 ± 0.06	0.14 ± 0.07
YNL274c	230 ± 36	36.8 ± 0.8
YIL074c	0.45 ± 0.10	0.09 ± 0.06
YGL185c	5.13 ± 1.66	2.25 ± 0.78
YER081w	0.47 ± 0.07	0.35 ± 0.05
Empty vector	0.68 ± 0.15	0.23 ± 0.18

Either glyoxylate (25 mM) or hydroxypyruvate (25 mM) were used as the substrate in the activity assay. The activities were measured as duplicates from two independent transformants and from two or three independent cultivations. The error limits are calculated with 95% confidence (±1.96*SEM).

The highest activity, an increase of approximately 350-fold on glyoxylate and 160-fold on hydroxypyruvate relative to the control, was measured in the *YNL274c* overexpressing strain. The ratio of the activity on glyoxylate to that on hydroxypyruvate in this strain was 6 : 1, which is similar to glyoxylate reductase isoenzyme I of *S. cerevisiae*, for which the measured activity towards hydroxypyruvate was 15% of that towards glyoxylate (Tochikura *et al*., 1979).

The activity on glyoxylate was also measured using NADH as a co-factor instead of NADPH, the latter being the preferred co-factor of glyoxylate reductase isoenzymes in *S. cerevisiae* (Fukuda *et al*., 1980; Tochikura *et al*., 1979). In the strain overexpressing ORF *YNL274c* the activity was 4% with NADH as a co-factor compared to that with NADPH (11 ± 1 and 230 ± 36 nkat/mg protein, respectively). However, when the ORF *YNL274c* was deleted, the activity with NADH, even though very low, was 25% of the activity on NADPH (0.05 ± 0.01 and 0.20 ± 0.10 nkat/mg protein, respectively).

The hydroxyisocaprotate dehydrogenase activity of ORF *YNL274c*

Since ORF *YNL274c* has been suggested to encode hydroxyisocaproate dehydrogenase, this activity was measured in the strains that showed elevated glyoxylate reductase activity and in the corresponding deletion strains, using NADPH as a co-factor (Table 3). The hydroxyisocaproate dehydrogenase activity was two- to seven-fold higher in the overexpression strains which had glyoxylate reductase activity, compared to the control strain, but it was considerably lower than the activity on glyoxylate or hydroxypyruvate (Table 2). In addition, the hydroxyisocaproate dehydrogenase activity was comparable in the strains overexpressing either ORF *YNL274c* or ORF *YPL113c*, around 0.5 nkat/mg protein, although the glyoxylate reductase activities of these strains differed considerably. In the strains with these two ORFs deleted, the activity was similar to the mother strain, BY4742. The

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Table 3. Hydroxyisocaproate dehydrogenase activity, nkat/mg total soluble protein, for the three homologous ORFs of glyoxylate reductase which had the highest activities with glyoxylate and hydroxypyruvate

	overexpression	deletion
YNL274c	0.49 ± 0.02	0.06 ± 0.01
YPL113c	0.55 ± 0.04	0.07 ± 0.01
YGL185c	0.15 ± 0.02	0.06 ± 0.01
Empty vector	0.07 ± 0.01	
BY4742		0.07 ± 0.01

A final concentration of 6 mM 2-oxoisocaproate was used in the assay. The activities were measured as duplicates from two or three independent cultivations and the error limits are calculated with 95% confidence (±1.96*SEM).

hydroxyisocaproate dehydrogenase activity using NADH as a co-factor was below the measurement limit (data not shown).

Deletion of ORF *YNL274c* leads to higher biomass concentration after diauxic shift

The CEN.PK2-1D yeast strain with either the empty expression vector or the expression vector containing ORF *YNL274c* under the *PGK1* promoter, the BY4742 yeast strain and BY4742 strain with a deletion of ORF *YNL274c* were grown in liquid medium on glucose (Figure 2A,B). The maximum specific growth rates of the strains on glucose were similar, 0.35/h for the CEN.PK2-1D strains and 0.32/h for the BY4742 strains. The strain overexpressing ORF *YNL274c* and its control strain, with the empty expression vector, grew similarly also during and after the diauxic shift. The strain in which ORF *YNL274c* was deleted reached the same biomass concentration ($OD_{600} = 6.2 \pm 0.2$) on glucose as its parent strain. However, during the first 3 h after the glucose had been consumed, the deletion strain accumulated a higher biomass concentration ($OD_{600} = 7.5 \pm 0.2$) than its parent strain ($OD_{600} = 6.5 \pm 0.2$) and continued to produce more biomass till the end of the cultivation. OD_{600} measurements 70 h after the glucose had been depleted were 9.0 ± 0.5 and 12.0 ± 0.5 for the parent strain, BY4742, and the deletion strain of ORF *YNL274c*, respectively. Ethanol produced from glucose was utilized to a comparable extent in both the deletion and control strain, within the 100 h studied (data not shown). When the four strains were grown on the oxidative carbon sources ethanol or glycerol, no difference in biomass concentration was observed compared to the control upon either deletion or overexpression of the ORF *YNL274c* (data not shown).

Glyoxylate reductase activity in the strain BY4742 and in deletion strain of ORF *YNL274c* was also measured during the cultivations (Figure 2C). In strain BY4742 the activity (0.2 ± 0.1) increased steadily during the exponential growth phase on glucose, and continued to rise

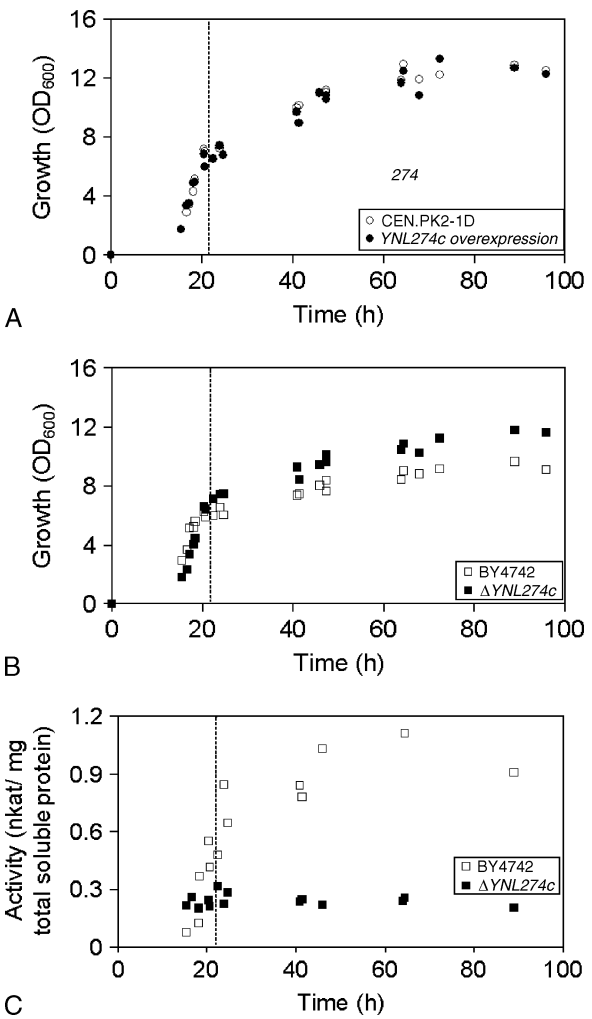

Figure 2. Cultivation on YSC medium, pH 5, with 20 g/l glucose. The dotted line indicates the time of glucose depletion in the cultures. The data are obtained from two independent cultivations. (A) Growth of CEN.PK2-1D strain overexpressing *YNL274c* and CEN.PK2-1D strain with an empty expression vector. (B) Growth of the deletion strain of *YNL274* and its parent strain BY4742. (C) Glyoxylate reductase activities of the deletion strain of *YNL274c* and its parent strain BY4742

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after the diauxic shift, reaching a value of ca. 1.0 nkat/mg protein at the end of the cultivation. However, the activity in the deletion strain remained around 0.2 nkat/mg protein throughout the entire cultivation.

Discussion

ORF *YNL274c* encodes a NADPH-dependent glyoxylate reductase, not a NADH-dependent hydroxyisocaproate dehydrogenase as was previously suggested. Therefore, ORF *YNL274c* has been renamed *GOR1* (glyoxylate reductase). On the basis of sequence homology, ORF *YNL274c* had previously been identified as one of three candidates to encode a NADH-dependent hydroxyisocaproate dehydrogenase. The two other candidates have been identified as phosphoglycerate dehydrogenases, *SER3* and *SER33*. It is worth noting that even though overexpression of ORF *YNL274c* resulted in increased activity towards α-ketoisocaproate, deletion of this ORF did not decrease the activity observed in the parental strain. The same was observed for ORF *YPL113c* and *YGL185c.* Thus, even though hydroxyisocaproate can act as a substrate for the enzyme products of these three ORFs, it is clearly a minor activity of these enzymes. The gene, if any, encoding a major NADH-dependent hydroxyisocaproate in *S. cerevisiae* remains to be identified.

ORFs *YPL113c* and *YGL185c,* which show similarity to hydroxyacid dehydrogenases, were shown to have activity towards glyoxylate and hydroxypyruvate. In addition, they had activity towards hydroxyisocaproate. However, whether the proteins encoded by these two ORFs are the only ones responsible for the observed NADPH-dependent glyoxylate reductase activity in the strain with the ORF *YNL274c* deleted remains to be determined. There also seems to be an NADH-dependent enzyme(s) with activity towards glyoxylate since the ratio between NADH- and NADPH-dependent activities was considerably higher in the deletion strain of *YNL274c* than in the other strains.

The NADPH needed for biosynthesis is produced by the oxidative branch of the pentose phosphate pathway and by aldehyde dehydrogenase, encoded by *ALD6* (Grabowska and Chelstowska, 2003; Minard and McAlister-Henn, 2005). NADPH is also required for antioxidation involving glutathione and/or thioredoxin, especially during oxidative metabolism. In comparison to the parental strain, the deletion of ORF *YNL274c* resulted in 75% lower glyoxylate reductase activity and in 30% higher biomass concentration after the diauxic shift. During the exponential growth phase on glucose there was no significant difference in either the glyoxylate reductase activity or growth. Hence, one may speculate that after the diauxic shift, the deletion of ORF *YNL274c* leads to a situation where the NADPH that the enzyme encoded by this ORF, i.e. *GOR1* would have used, is now available for other reactions, such as biomass production. In support of this, it has been recently shown that ORF *YNL274c* has a binding site for Stb5, a regulator of pentose phosphate pathway and NADPH production (Larochelle *et al.*, 2006). However, it remains an open question, why an initial glucose consumption phase would be needed to observe the enhanced biomass accumulation. Cultivation on ethanol or glycerol resulted in comparable biomass production with both the deletion and the parental strain.

High levels of glyoxylate have been shown to be formed during growth of *S. cerevisiae* on glucose. Glyoxylate is formed by isocitrate lyase from isocitrate and by a deaminase from glycine (Villas-Bõas *et al.*, 2005a, 2005b; Devantier *et al.*, 2005). It is consumed by malate synthase, alanine : glyoxylate aminotransferase and glyoxylate reductase (Figure 3). The biological role of glyoxylate reductase in *S. cerevisiae* remains unknown but, as the activity is induced once glucose is depleted, it is tempting to speculate that it functions in

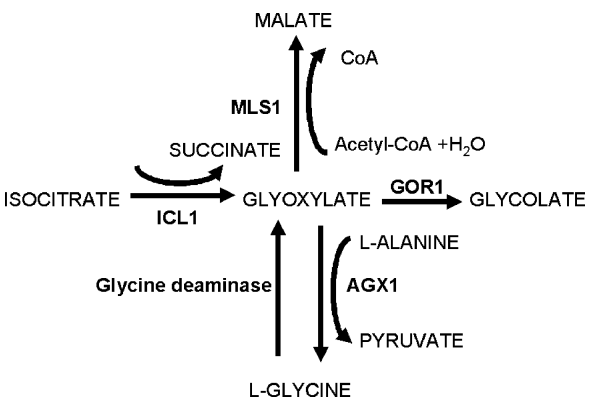

Figure 3. Glyoxylate metabolism in *Saccharomyces cerevisiae.* The gene encoding glycine deaminase is not known

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combination with the glyoxylate cycle or in the direction of the carbon flux to gluconeogenesis, as has been suggested in higher eukaryotes (Holmes and Assimos, 1998). Furthermore, since glyoxylate is toxic for organisms (Poldelski *et al*., 2001; Zabrodskii *et al*., 2002), activities that are able to convert glyoxylate to less toxic substances are needed under conditions where this acid is formed.

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