The effect of iron limitation on glycerol production and expression of the isogenes for NAD$^+$-dependent glycerol 3-phosphate dehydrogenase in *Saccharomyces cerevisiae*

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Abstract When deprived of iron, *Saccharomyces cerevisiae* rearranges its metabolic flux towards increased glycerol production. This work examines the role and regulation of *GPD1* and *GPD2*, encoding two isoforms of glycerol 3-phosphate dehydrogenase, in glycerol production during iron starvation. The two genes respond differently on transfer of cells to iron-limited conditions. Whereas the expression of *GPD2* increases about 3-fold, that of *GPD1* does not exhibit significant changes. Deletion of either *GPD1* or *GPD2* alters the capacity for glycerol production during iron-limited as well as iron sufficient conditions. However, loss of function of either gene does not seem to provoke compensatory flux via the other gene product. As judged from the glycerol production, the amount produced by each single mutant adds approximately up to the level produced by the parental strain. In agreement with the pattern of expression of *GPD2*, this gene product was estimated to account for the bulk of the glycerol production (about 60%) during iron-limited conditions. The strong growth inhibition caused by iron starvation was reversed by the addition of iron also for a *gpd1*Δ*gpd2*Δ double deletion mutant, which is unable to produce any detectable glycerol.

Key words: Yeast; Iron limitation; Glycerol production; *GPD2* gene expression; *Saccharomyces cerevisiae*

1. Introduction

Iron is essential for all eukaryotic cells and most prokaryotes [1,2], playing a crucial role for the activity of heme-containing proteins and redox-active metalloenzymes. Although iron is a highly abundant metal, it is difficult to capture since environments containing oxygen readily oxidise iron from the ferrous (Fe^{2+}) to ferric (Fe^{3+}) state [3], leading to formation of insoluble polymeric hydroxides [2]. Moreover, since iron can generate deleterious oxidising radicals when present in excess [4], the intracellular iron concentration has to be maintained within a tightly controlled range.

To mobilise external iron, many microorganisms synthesise and excrete iron-chelating siderophores that have a high affinity for ferric iron. *Saccharomyces cerevisiae* does, however, not produce siderophores [5] and has to satisfy its iron needs by alternative strategies, that is by reductive iron assimilation [1,6] or uptake of siderophores produced by other organisms [7]. Reductive uptake of iron occurs in two steps. First, extracellular Fe^{3+} is reduced to Fe^{2+} by membrane bound Fe^{3+} reductases, products of the *FRE1* [8] and *FRE2* [9] genes, followed by ferrous uptake via one of two transport systems for Fe^{2+}. The high affinity transport system involves a multicopper oxidase, encoded by *FET3* [10], and a permease, encoded by the *FTR1* gene [11], while the low affinity system is dependent on the *FET4* gene [12]. Cultured under iron-limited conditions, *S. cerevisiae* changes its pattern of metabolism. An immediate consequence of cellular iron depletion appears to be malfunction of the respiratory chain, which contains several iron-dependent enzymes [13]. This respiratory insufficiency might lead to accumulation of NADH both in the cytosol and in mitochondria. It was demonstrated by Krieger and Ernst [14] that the stability of the messenger RNAs for genes encoding glyceraldehyde 3-phosphate dehydrogenase (*TDH3*) and triosephosphate isomerase (*TPI1*) are reduced more than 3-fold during iron-limited conditions. This was assumed to lead to a decreased flux through glycolysis downstream of these enzymes and an increase in the dihydroxyacetone phosphate pool, explaining an observed increase in glycerol production during iron limitation. Glycerol is produced by reduction of dihydroxyacetone phosphate to glycerol 3-phosphate in an NADH-coupled oxidation (Fig. 1), followed by de-phosphorylation of glycerol 3-phosphate. The first reaction is catalysed by two isoforms of glycerol 3-phosphate dehydrogenase product of the *GPD1* and *GDP2* isogenes [15,16], while the subsequent step is carried out by the action of a glycerol 3-phosphatase, encoded by the two isogenes *GPP1* and *GPP2* [17]. The intracellular Gpd1p level is subject to control by environmental osmolarity [18], involving transcriptional control of the *GPD1* gene via the osmosignalling high osmolarity glycerol pathway [19,20]. Expression of *GPD2* appears, on the other hand, to be controlled by the redox status of the cytosol, conditions that cause accumulation of NADH promote induction of *GPD2* [21]. Deletion of both *GPD1* and *GPD2* leads to abolished glycerol production, conferring severe osmosensitivity and arrested anaerobic growth due to inability to oxidise the excess NADH generated under anoxic conditions [21]. In the present study, we have examined the role of *GPD1* and *GPD2* in the stimulated production of glycerol during iron-limited growth conditions.

2. Materials and methods

2.1. Yeast strains and growth conditions

The *S. cerevisiae* strains used in this study are described in Table 1. Cells were grown at 30°C in defined minimal medium, containing trace metals (with or without $FeSO_4$), vitamins, $(NH_4)_2SO_4$, KH_2PO_4 and $MgSO_4$ as described by Verduyn et al. [22], supplemented with 2% glucose and 120 μg/ml of required amino acids or nucleosides. To

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ensure iron-deprived conditions, the water used for the iron free medium was extracted twice with chloroform containing 3% (w/v) 8-hydroxychinoline and twice with pure chloroform before use. The salt solutions were extracted with chloroform after salt addition. Traces of chloroform were removed by repeated boiling and shaking. Solutions of vitamins, amino acids/nucleosides were filter-sterilised, whereas all other solutions were autoclaved with tightened lids. All glassware was washed repeatedly with 5 M HCl and rinsed with chloroform/8-hydroxychinoline-treated water to near neutral pH. Plastic tubes were rinsed once in 1 M NaOH, dissolved in iron free water.

2.2. Northern blotting

Cell cultures of 25 ml were removed and total RNA was isolated according to standard protocols [23]. The RNA quality was checked on ethidium bromide-stained agarose gels (1% w/v) and the samples quantified by spectrophotometry (Beckman DU65). Samples containing 15 μg of total RNA were denatured (5 min, 65°C), run on 1% low formaldehyde (2.5% v/v) agarose gels at 10 V/cm for 75 min and then checked for quality on UV-illuminated 254 nm TLC plates (kindly provided by J.C. Ulvinge, AB Astra/Hässle, Sweden). The gels were blotted overnight to positively charged nylon membranes (Boehringer Mannheim, Germany) by capillary transfer using diethylpolycarbonate-treated 10×SSC [23] as transfer buffer. Blotted filters were cross-linked by 1 min exposure to low wavelength UV and baked at 80°C for 2 h.

Pre-hybridisations were performed for 3–4 h at 55°C in 5×SSC [23], 10 mM sodium phosphate (pH 6.5), 10×Denhardt's solution [23], 2% sodium dodecyl sulphate (SDS) and 100 mg/ml herring sperm DNA. Hybridisations were performed at 55°C with the same solution supplemented with 10% PEG 4000 and labelled oligonucleotides (5 ng/ml) for 15–20 h. The filters were washed in 1×SSC/1% SDS, twice at room temperature for 10 min and once at the hybridisation temperature for 5 min. The filters were scanned and analysed using a phosphorimager (Molecular Dynamics, USA). Membranes were stripped by shaking in sterile water at 80°C for 10 min. An actin (*ACT1*) oligonucleotide acted as an internal control of the RNA level. The oligonucleotides were 5′-labelled using 25 μCi [γ-^{32}P]ATP (Amersham, UK) and 5 U poly-nucleotide kinase (Boehringer Mannheim, Germany) per 50 ng probe left at 37°C for 30 min. Unincorporated ATP was displaced using a Sephadex G50 (Pharmacia, Sweden) mini column. The sequences of oligonucleotides used were: *GPD1*: 5′-TGTACTATTGGAGCGAAAACTTCT-3′, *GPD2*: 5′-GGTCCTCATGACAGTGTTTGTGCT-3′, *ACT1*: 5′-AATCGATTCTCAAAATGGCGTGAGG-3′.

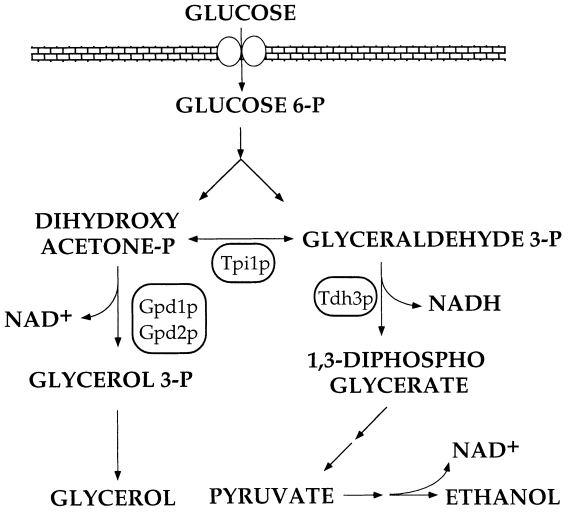

Fig. 1. Outflow of the glycerol biosynthetic pathway from glycolysis in *S. cerevisiae*. Abbreviations: Gpd1p and Gpd2p, isoforms of NAD-dependent glycerol 3-phosphate dehydrogenase. Tpi1p, triosephosphate isomerase. Tdh3p, major isoform of NAD-dependent glyceraldehyde 3-phosphate dehydrogenase.

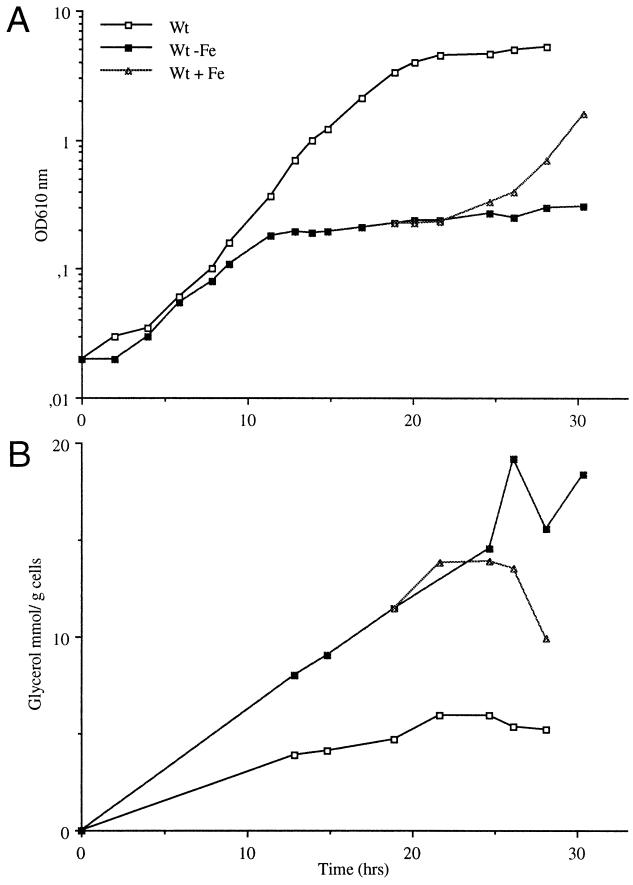

Fig. 2. Growth (A) and glycerol production (B) of *S. cerevisiae* W303-1A strain cultured in the iron sufficient (□) and iron-limited (■) or after addition of iron (0.6 g/l $FeSO_4$, added at 18 h) to an iron-limited culture (Δ).

2.3. Glycerol analysis

For analysis of total (intracellular plus extracellular) glycerol, samples of 1.5 ml were removed from growing cultures and boiled for 10 min, after which the cell debris was pelleted and discarded. Glycerol was determined using a commercial kit (Boehringer Mannheim, Germany). The amount of glycerol was correlated to the optical density (OD) (cell mass) of the cultures at time of harvest (one OD unit at 610 nm = 0.3 g dry weight cells/l) and expressed as mmol glycerol/g cells dry weight.

3. Results

3.1. Effect of iron limitation on cell growth and glycerol production

To examine the cellular response to iron limitation, the *S. cerevisiae* strain W303-1A was cultured overnight in defined minimal medium and inoculated to flasks containing fresh iron sufficient or iron-limited medium (Fig. 2A). While cells of the iron replete culture maintained a generation time of 2.1 h during the exponential growth phase, the growth rate of the iron-limited culture was gradually decreased to become severely inhibited 8–10 h after transfer to the fresh medium. This inhibition was clearly linked to iron limitation, since growth was recovered after external addition of iron. Analysis of total glycerol production (Fig. 2B) confirmed that iron limiting conditions affect glycerol production, the rate of glycerol synthesis was several fold increased by iron starvation.

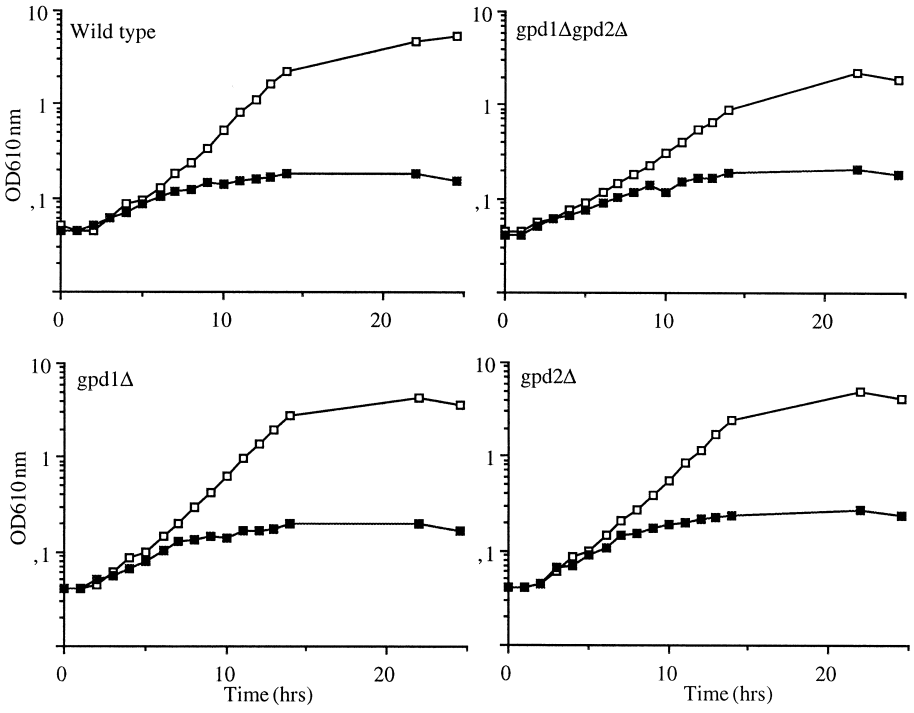

Fig. 3. Growth of *S. cerevisiae* W303-1A parental strain, the *gpd1*Δ and *gpd2*Δ single mutants and the *gpd1*Δ*gpd1*Δ double mutant when cultured in iron sufficient (□) and iron-limited (■) medium.

Importantly, external addition of Fe^{2+} to the iron-depleted cells caused a rapid drop in the relative rate of glycerol production, strongly supporting that the iron status of the cells affects glycerol production.

3.2. Role of GPD1 and GPD2 in glycerol biosynthesis under iron limitation

To examine the role of the two isogenes *GPD1* and *GPD2*, encoding NAD^+-dependent glycerol 3-phosphate dehydrogenase, for glycerol production under iron-limited conditions, we examined the growth behaviour (Fig. 3) and glycerol production (Fig. 4) of mutants deleted for each or both of these genes. Both the *gpd1*Δ and *gpd2*Δ mutants showed a growth behaviour that was similar to that of the parental W303-1A strain under iron sufficient as well as iron-limited conditions. In both the *gpd1*Δ and *gpd2*Δ mutants, glycerol production increased under iron starvation, although not as much as in the parental strain (Fig. 4). In fact, the glycerol produced by each mutant adds approximately up to the glycerol production of the parent strain, indicating that neither of the gene deletions brings about a compensatory expression/activity of the remaining isogene product. As inferred from the glycerol production by the *gpd1*Δ and *gpd2*Δ mutants, the glycerol produced via the Gpd2p enzyme increases markedly during iron starvation to constitute about 60% of the total glycerol in a strongly iron-starved culture (14 h, Fig. 4). We also ex-

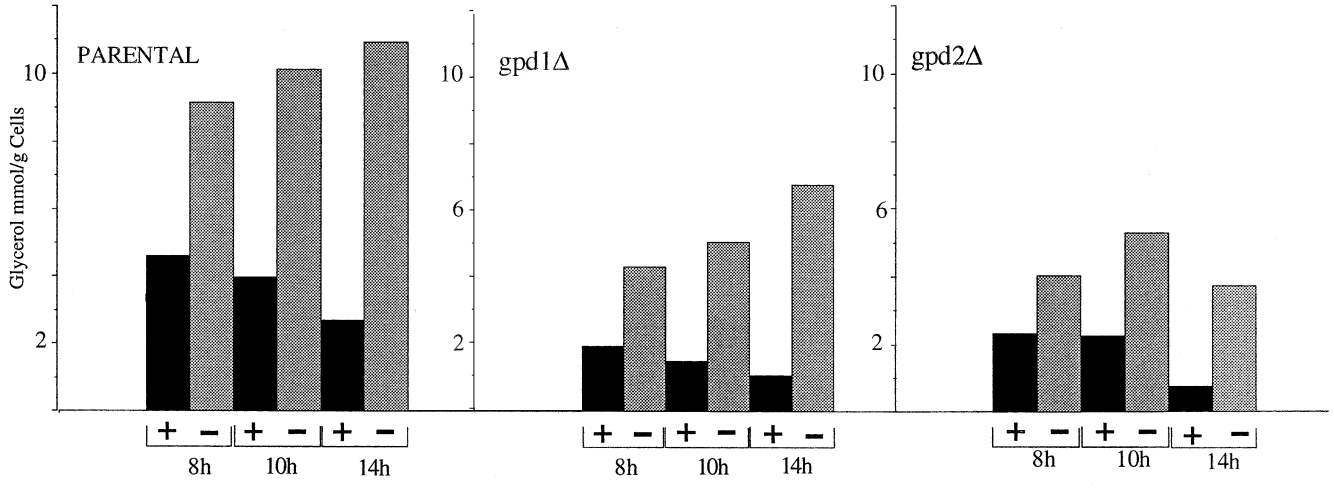

Fig. 4. Total glycerol production by the W303-1A parental strain and the *gpd1*Δ and *gpd2*Δ single mutants when cultured in iron sufficient (+) and iron-limited (−) medium. Cells were cultured as described in Fig. 1 and samples for glycerol determination were taken 8, 10 and 14 h after inoculation, as indicated. The *gpd1*Δ*gpd2*Δ mutant did not produce any detectable glycerol during the conditions tested (data not shown).

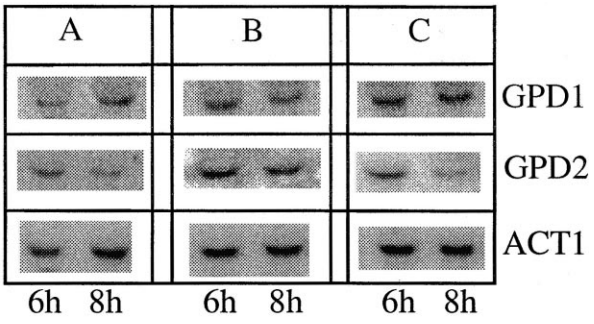

Fig. 5. Northern analysis of *GPD1* and *GPD2* expression in W303-1A parental cultured in (A) iron sufficient and (B) iron-depleted cultures and (C) after transfer of an iron-starved overnight culture to iron sufficient medium. The times indicated denote hours after inoculation/transfer. *ACT1* expression was used as a control.

amined the behaviour of the *gpd1*Δ*gpd2*Δ double mutant during iron starvation. Since this mutant is unable to produce glycerol in significant amounts [21], it remained possible that the cells would not survive long-time exposure to iron limitation. However, still after 24 h of iron starvation, a *gpd1*Δ*gpd2*Δ culture regained growth on addition of $FeSO_4$ (data not shown), indicating that the putative intracellular NADH accumulation of iron-starved cells does not cause any lasting damage on the metabolic machinery.

3.3. Effect of iron limitation on expression of GPD1 and GPD2

To study any iron-mediated regulation of the *GPD1* and *GPD2* genes, their expression was examined by Northern analysis of RNA extracted from cells incubated under iron replete and iron deplete conditions (Fig. 5). While iron availability did not significantly affect the expression of the *GPD1* gene, that of *GPD2* increased about 3-fold under iron limitation, which agrees well with the observed increase of Gpd2p-mediated glycerol production under iron starvation (Fig. 4). Furthermore, transfer of cells from iron-limited to iron sufficient medium resulted in a pronounced decrease of *GPD2* expression while *GPD1* remained unaffected. These results demonstrate that *S. cerevisiae* responds to iron limitation by increased expression of one of the isoenzymes in the glycerol biosynthesis pathway.

4. Discussion

This work confirms that exposure of *S. cerevisiae* to iron starvation leads to increased glycerol production and shows that *GPD2*, one of the two isogenes encoding NAD-dependent glycerol 3-phosphate dehydrogenase, is induced by iron limiting conditions. One of the most vulnerable targets for iron deprivation is the respiratory chain. Iron is required for heme biosynthesis and for the activity of many heme proteins and non-heme iron proteins which participate in electron transfer reactions in the respiratory chain [24]. Hence, iron limitation will decrease or disrupt the respiratory functions, thereby imposing physiological conditions that mimic those of anaerobicity or lowered availability of oxygen. Therefore, iron limitation is likely to reduce the capacity of the respiratory apparatus to oxidise cytosolic and mitochondrial NADH, one of the main suppliers of reducing equivalents to the respiratory chain. We have previously demonstrated that *S. cerevisiae*, in the absence of oxygen, has no endogenous alternative for oxidation of NADH than reduction of dihydroxyacetone phosphate to glycerol 3-phosphate, via the NAD^+-linked glycerol 3-phosphate dehydrogenase, ultimately resulting in glycerol production [21]. In agreement with the increased expression of *GPD2* in cells deprived of iron (Fig. 5), the production of glycerol by *gpd* mutants (Fig. 4) implies that the *GPD2*-encoded dehydrogenase increases its contribution to glycerol synthesis under iron limitation, to become the isozyme that mediates most of the glycerol production. There are, however, other important mechanisms that may re-direct the glycolytic flux towards increased glycerol production in iron insufficient cells. The stability of the transcripts for triosephosphate dehydrogenase (Tpilp) and glyceraldehyde 3-phosphate dehydrogenase (Tdh3p) is regulated by the ambient iron concentration and as a result, the levels fall about 3-fold on iron deprivation [14]. Reduced levels of these enzymes might provoke accumulation of dihydroxyacetone phosphate since the kinetics for isomerisation of dihydroxyacetone phosphate and glyceraldehyde 3-phosphate (Fig. 1) will be constrained, as will the subsequent NAD^+-coupled oxidation of glyceraldehyde 3-phosphate to glycerate 1,3-diphosphate. The concerted effects of all these changes are expected to serve the purpose of generating an increased flux to glycerol, the anoxic redox sink of *S. cerevisiae*. The transcriptional induction of *GPD2* during anaerobic conditions was demonstrated to be independent of *ROX1* and *ROX3*, encoding transcription factors involved in hypoxic gene control and global stress response, respectively [25,26]. Since *GPD2* is induced also by iron starvation under fully aerobic conditions (Fig. 5), the regulatory mechanism appears insensitive to the ambient oxygen concentration. This is in accordance with the observation that *GPD2* is induced in aerobically grown cells treated with bisulfite [21]. Bisulfite forms a complex with acetaldehyde and prevents its reduction to ethanol by alcohol dehydrogenase, leading to NADH accumulation in the cell [27]. It thus appears that *GPD2* has a key role for the redox-mediated glycerol production in *S. cerevisiae* and that the transcriptional activation of this gene may be directly or indirectly controlled by the NAD^+/NADH ratio in the cell. Such a signal might be responsible for eliciting the observed induction of *GPD2* under iron-limited conditions. No significant iron-dependent response was observed for *GPD1*, the other isoform of the cytosolic glycerol 3-phosphate dehydrogenase, in accordance with the osmostress-controlled regulation of this gene, which mediates the osmoregulatory glycerol response and remains unaffected by transfer to anaerobic conditions [21].

The bioavailability of iron is paradoxically low since the oxidised form (Fe^{3+}) forms extremely insoluble hydroxides [2]. Hence, iron limitation is a frequently encountered condition in most natural environments. *S. cerevisiae*, which does

Table 1
Strains used in present study

Strain[a]	Genotype
YSH6.142-3A	Mat α
YSH6.142-3B	Mat a *gpd1Δ::TRP1*
YSH6.142-3C	Mat a *gpd2Δ::URA3*
YSH6.142-3D	Mat α *gpd1Δ::TRP1gpd2Δ::URA3*

These strains harbour the following additional mutations: *leu2-3/112 ura3-1 trp1-1 his3-11/15 ade2-1 can1-100 GAL SUC2 mal*^{0}.
[a]These strains are derivatives of W303-1A [28] and have previously been described [21].

not secrete iron-scavenging siderophores, relies mainly on enzymatic reduction of ferric iron at the cell surface to make iron available for uptake. This possibly makes yeast cells sensitive to iron limitation, raising requirements for alternative adaptive mechanisms. An intricate regulation of central metabolism to adjust the intracellular redox balance by glycerol production during iron limitation may be an important component of these adaptive responses.

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