A transcriptome analysis of isoamyl alcohol-induced filamentation in yeast reveals a novel role for Gre2p as isovaleraldehyde reductase

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Received 10 March 2006; revised 22 May 2006; accepted 13 June 2006.
First Published online 25 September 2006.

DOI:10.1111/j.1567-1364.2006.00151.x

Editor: Ian Dawes

Keywords
Saccharomyces cerevisiae; isoamyl alcohol-induced filamentation; *DLD3*; *GRE2*; D-lactate dehydrogenase; isovaleraldehyde reductase.

Abstract

A transcriptome analysis was performed of *Saccharomyces cerevisiae* undergoing isoamyl alcohol-induced filament formation. In the crucial first 5 h of this process, only four mRNA species displayed strong and statistically significant increases in their levels of more than 10-fold. Two of these (YEL071w/*DLD3* and YOL151w/*GRE2*) appear to play important roles in filamentation. The biochemical activities ascribed to these two genes (D-lactate dehydrogenase and methylglyoxal reductase, respectively) displayed similarly timed increases to those of their respective mRNAs. Mutants carrying *dld3* mutations displayed reduced filamentation in 0.5% isoamyl alcohol and needed a higher concentration of isoamyl alcohol to effect more complete filament formation. Hence, *DLD3* seems to be required for a full response to isoamyl alcohol, but is not absolutely essential for it. Mutants carrying *gre2* mutations were derepressed for filament formation and formed large, invasive filaments even in the absence of isoamyl alcohol. These results indicate a previously unsuspected and novel role for the *GRE2* gene product as a suppressor of filamentation by virtue of encoding isovaleraldehyde reductase activity.

Introduction

The yeast *Saccharomyces cerevisiae*, which is well known as a 'budding yeast', can undergo changes in replicative patterns and morphologies to produce filamentous elongated cells called 'pseudohyphae' in diploids and 'invasive filaments' in haploids (Dickinson, 2004). This switch to filament formation has been studied both for its intrinsic interest and also as a model for related processes in other less tractable yeast species, such as the clinically significant *Candida albicans*. Filamentation in *S. cerevisiae* can be induced by a variety of environmental conditions, including nitrogen starvation or limitation (Rua *et al*., 2001), growth on a poor nitrogen source (Dickinson, 1994) or growth in the presence of low concentrations of fusel alcohols (Dickinson, 1996; Lorenz *et al*., 2000; La Vall & Wittenberg, 2001). Fusel alcohols are the products of amino acid catabolism, and accumulate when nutrients become limiting (Dickinson *et al*., 1997, 1998, 2000, 2003). Thus, yeast responds to its own metabolic byproducts.

To date, much of the work on filamentation in yeast has focused on the signalling pathways and alterations to the cell

cycle that evoke the morphological changes involved (for reviews see Rua *et al*., 2001; Dickinson, 2004). Two signalling pathways have been recognized: a MAP kinase pathway, which comprises elements of the mating signal transduction pathway, and a cAMP signalling pathway. The key feature is that both signalling pathways converge to control expression of the cell wall flocculin Flo11, which is a glycerol phosphoinositol-anchored cell surface protein required for the adhesion of mothers to daughters, and for substrate binding and penetration. One of the most significant alterations to the cell cycle of pseudohyphal cells is the extension of G2 phase, which results in daughters being larger than those of yeast-form cells and equal in size to their mothers (Kron *et al*., 1994). Despite it having been demonstrated that isoamyl alcohol induces the Swe1-dependent morphogenesis checkpoint (Martinez-Anaya *et al*., 2003), and affects translation by binding to the eIF2B initiation factor (Ashe *et al*., 2001), little else is known about the biochemical basis of isoamyl alcohol-induced filament formation. We have sought to define the cell biological and biochemical changes that accompany isoamyl alcohol-induced filament formation, and we have observed that filaments contain significantly greater mitochondrial mass and an increased chitin content in comparison with yeast-form cells (Kern *et al*., 2004). Unlike the situation in yeast-form cells, the chitin of filaments is not confined to bud scars and the chitin ring between mother and daughter cells, but is distributed over the majority of the cell surface. Hence, the walls of filaments have greater strength and rigidity than those of yeast-form cells, representing an important advantage to the filaments, which can penetrate solid media (Kern *et al*., 2004).

In an attempt to gain further insight into the important cell biological and biochemical events of filamentation, we have examined the global transcriptional response of *S. cerevisiae* to isoamyl alcohol. The aim was to identify those genes whose expression increased very significantly in the presence of this fusel alcohol, with a view to examining their roles in the response to the fusel alcohol. There is also the potential to identify functions for genes whose roles are currently unknown by virtue of their association with filamentation. We report this transcriptome analysis and focus upon two genes whose expression increased very significantly over the first 5 h of filament formation: *DLD3* (YEL071w) and *GRE2* (YOL151w). The data indicate a previously unsuspected and novel role for the *GRE2* gene product as a suppressor of filament formation.

Materials and methods

Yeast strains, media and cultural conditions

The strains of *S. cerevisiae* used are described in Table 1. Strain IWD72 is the prototrophic wild-type strain, which we have employed previously for studies of both fusel alcohol formation and the morphological response to fusel alcohols (Dickinson, 1996; Dickinson *et al*., 1997, 1998, 2000, 2003). Strain BY4741 is the parental strain of the *Saccharomyces* Genome Deletion Project. It is the parent of strains Y00313 and Y07320. As our preliminary studies have repeatedly shown that strain BY4741 responds very poorly to isoamyl alcohol, it might be difficult to interpret the effects of any mutations on filamentation in this genetic background. Hence, we crossed Y00313 and Y07320 to wild-type strain IWD72 to produce strain 70.1.1 (mutated in *DLD3*) and strains 71.1.2, 71.5.9, and 71.5.10, which are all mutated in *GRE2*. Standard genetic techniques were used in both crosses (Mortimer & Hawthorn, 1975; Sherman, 1975). Strain PHY14 was produced by transforming strain 71.5.9 with plasmid pYCG_YOL151w, which is based on plasmid pRS416 (Sikorski and Hieter, 1989) and carries the cognate gene *GRE2*. The transformation was performed essentially as described by Ito *et al*. (1983). As *URA3* is the selectable marker on plasmid pCG_YOL151w, transformants were initially sought on glucose minimal medium lacking uracil and subsequently confirmed by isolation of the plasmid from putative transformants.

Table 1. Yeast strains used

Strain	Genotype	Source or reference
IWD72	MATα	Dickinson (1996)
L5528	*MAT***a**, *ura3-52*, *his3::hisG*	Roberts & Fink (1994)
BY4741	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	EUROSCARF*
Y00313	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 dld3::KANMX4*	EUROSCARF*
Y07320	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 gre2::KANMX4*	EUROSCARF*
70.1.1	*MATα ura3Δ0 dld3::KANMX4*	This work
71.1.2	*MAT***a** *leu2Δ0 gre2::KANMX4*	This work
71.5.9	*MATα ura3Δ0 gre2::KANMX4*	This work
PHY14	*MATα ura3Δ0 gre2::KANMX4* /pYCG_YOL151W	This work
71.5.10	*MAT***a** *gre2::KANMX4*	This work

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Strains were grown in YEPD medium, which comprised 1% (w/v) Difco yeast extract (Becton-Dickinson), 2% (w/v) Difco Bacto peptone (Becton-Dickinson), 2% (w/v) glucose, 0.01% (w/v) adenine and 0.01% uracil. The cells were cultured in a gyrorotatory shaker (160 r.p.m) at 30 °C in conical flasks filled to 40% nominal capacity. Filamentation was induced by growing the cells to an $OD_{600\,nm}$ of 0.6 and then adding isoamyl alcohol to a final concentration of 0.5% (v/v). For studies of invasion, small patches of cells (*c*.

10×3 mm) were transferred to the surface of solid YEPD medium [which included 2% (w/v) agar] using sterile velvet cloth. At various times thereafter, the surface growth of yeast was removed by gently rinsing in a fine stream of distilled water. Then, a block of agar where the yeast had been growing was removed using a sterile scalpel and inspected microscopically for the presence of invasive cells.

Transcript profiling

Saccharomyces cerevisiae L5528 cells were grown at 30 °C in YPED to exponential phase, and then split into two: 0.5% (v/v, final concentration) isoamyl alcohol was added to one half, and the other half was the control. RNA was isolated after 5 and 15 h, and ^{33}P-labelled hybridization probes were made using standard procedures (Hauser *et al.*, 1998). Hybridizations were performed with *S. cerevisiae* arrays, and the signals detected using a FUJI FLA-3000 phosphorimager as described previously (Yin *et al.*, 2003). Phosphorimages were analysed using ARRAY VISION software (Amersham, Buckinghamshire, UK), and data quality assessment, normalization and statistical analyses of independent replicate experiments were performed with the M-CHIPS software package (Fellenberg *et al.*, 2001, 2002). Fold regulation in isoamyl alcohol-treated cells was measured relative to the corresponding untreated control cells. The complete transcript profiling dataset is available at http://mips.gsf.de/proj/eurofan/eurofan_2/b2/. Functional categories were assigned using the MIPS catalogue (http://mips.gsf.de/proj/yeast/CYGD/db/catalog_index.html).

Enzyme assays

Cells were harvested by centrifugation at the growth temperature. The growth medium was decanted, and the cell pellet was resuspended in 1 mL of ice-cold buffer A (50 mM sodium phosphate buffer, pH 7.4, containing 2 mM 2-mercaptoethanol and 2 mM EDTA). The cells were broken by agitating at top speed for 35 s with an equal volume of acid-washed 40-mesh glass beads in a Braun MSK homogenizer. The homogenate was separated from the glass beads and centrifuged at 11 600 ***g*** for 12.5 s. The clearer supernatant was used immediately in the enzyme assays.

D-Lactate ferricytochrome *c* oxidoreductase was assayed essentially as described by Chelstowska *et al.* (1999). The assay comprised 830 μL of 50 mM sodium phosphate buffer (pH 8.0), phenazine methosulphate (30 nmol) and 2,6-dichloroindophenol (50 nmol) in a total volume of 850 μL. The clarified cell homogenate was preincubated in the assay mixture for 3 min before initiation of the reaction with 100 μL (3 μmol) of D-lactate (lithium salt). Sodium phosphate buffer (pH 8.0) (100 μL) was added to the blank cuvette. The reaction was followed at 600 nm.

The assay for methylglyoxal reductase comprised 800 μL of 50 mM sodium phosphate buffer (pH 7.0) and NADPH (100 nmol) in a total volume of 900 μL. The clarified cell homogenate was preincubated in the assay mixture for 3 min before initiation of the reaction with 100 μL (10 μmol) of methylglyoxal. Sodium phosphate buffer (pH 7.0) (100 μL) was added to the blank cuvette. The reaction was followed at 340 nm. Assays of isovaleraldehyde reductase were carried out in a similar fashion, except that 10 μmol of isovaleraldehyde replaced methylglyoxal as substrate.

All enzyme activities were determined in duplicate at each time of sampling from two (isovaleraldehyde reductase) or three (D-lactate ferricytochrome *c* oxidoreductase and methylglyoxal reductase) independent cultures. Protein content was determined by the method of Bradford (1976) so that the enzyme activities could be converted to specific activities.

Microscopy and photography

Small aliquots of cells removed from liquid cultures or blocks of solid agar were examined using an Olympus BH2 microscope at $\times 40$ magnification. Photographs were taken using a Fujifilm Fujix digital camera, model HC-300Z. The images have not been enhanced or rendered in any way.

Results

Transcriptome analysis reveals key genes involved in the early responses to isoamyl alcohol

The complete dataset (acquired as part of the B2 node of the EUROFAN II project) is available at http://mips.gsf.de/proj/eurofan/eurofan_2b2/. Transcriptional changes have been colour-coded to denote statistical significance, using the following scheme: blue, very significant decrease; cyan, significant decrease; red, very significant increase; yellow, significant increase (Yin *et al.*, 2003). Since significant degrees of isoamyl alcohol-induced filamentation are attained by 5 h (Martinez-Anaya *et al.*, 2003), the present analysis focused only on those genes showing very significant increases in the presence of 0.5% isoamyl alcohol within the first 5 h. We have further sorted those genes that showed very significant increases by 5 h in isoamyl alcohol according to the magnitude of the transcriptional increase (Table 2). Only four ORFs displayed very significant increases in their RNA species by more than 10-fold. One of these, YOL150c, is described as being a dubious ORF and has no known function, so it was not considered further. YOR153w (*PDR5*) encodes a plasma membrane pump, which is induced in response to a wide range of environmental changes. The remaining two ORFs appeared to offer clear new insights into the molecular biology of filament formation, as explained below.

Table 2. Genes whose transcription showed a very significant increase of at least 10-fold by 5 h in YEPD medium containing 0.5% isoamyl alcohol

ORF	Gene name	Function	Fold increase
YEL071w	*DLD3*	Cytoplasmic D-lactate dehydrogenase	11.0
YOL150c		Dubious ORF. Function unknown	13.0
YOL151w	*GRE2*	NADPH-dependent methylglyoxal reductase	12.8
YOR153w	*PDR5*	Plasma membrane-located pump	10.0

YEL071w (*DLD3*) encodes a cytoplasmic D-lactate dehydrogenase (Chelstowska *et al.*, 1999). Furthermore, this gene is subject to the retrograde response (the phenomenon in which nuclear-encoded genes are regulated by the mitochondria.) YOL151w (*GRE2*) has been described as an NADPH-dependent methylglyoxal reductase (Chen *et al.*, 2003) (or D-lactaldehyde dehydrogenase, depending upon the direction in which the enzyme is considered to act). D-Lactate dehydrogenase and D-lactaldehyde dehydrogenase may operate adjacent to each other in a metabolic pathway, thereby offering the prospect of a coherent biochemical explanation for (at least part of) the metabolism associated with isoamyl alcohol-induced filamentation. Knowing that significantly more mitochondria are present during filamentation (Kern *et al.*, 2004), and that *DLD3* is subject to the retrograde response, we therefore focused upon the metabolic activities encoded by these two highly expressed genes.

The specific activities of D-lactate dehydrogenase and methylglyoxal reductase peak in the early stages of filamentation.

The specific activity of D-lactate dehydrogenase more than doubled during the first 5 h after the yeast had been transferred to medium containing 0.5% isoamyl alcohol (Fig. 1). Subsequently, the specific activity progressively declined, so that by 24 h it was only about one-fifth the level that was present in the original culture. The specific activity of methylglyoxal reductase doubled between 5 and 6 h after transfer to medium containing 0.5% isoamyl alcohol (Fig. 2). The specific activity then quickly declined to be undetectable at 8 h and then remained at very low levels for the remainder of the experiment. Therefore, both enzymes are elevated under conditions that induce their respective mRNA species.

Disruption of *DLD3* results in an impaired response to 0.5% isoamyl alcohol

Strain 70.1.1, which has a mutation in its *DLD3* gene, formed fewer filaments in the presence of 0.5% isoamyl alcohol than the wild-type strain IWD72 (Table 3). Increasing the isoamyl alcohol concentration to 0.6% partially restored filament formation in the mutant. Even so, the filaments that were formed in both 0.5% and 0.6% isoamyl alcohol were very small and thin (data not shown). Thus, *DLD3* seems to be required for a full response to isoamyl alcohol, but is not absolutely essential for it.

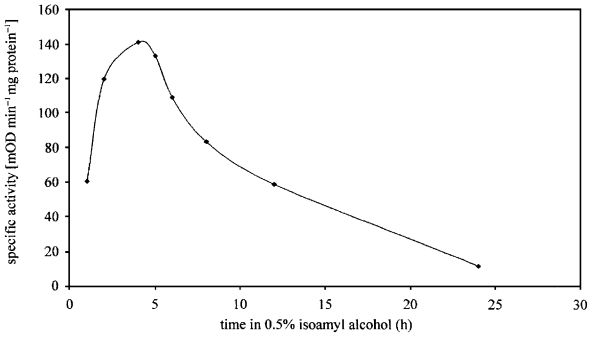

Fig. 1. D-Lactate dehydrogenase activity in wild-type strain IWD72 induced to undergo filamentation in liquid YEPD medium containing 0.5% isoamyl alcohol.

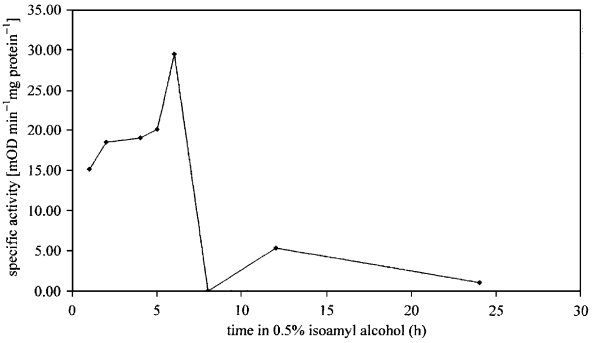

Fig. 2. Methylglyoxal reductase activity in wild-type strain IWD72 induced to undergo filamentation in liquid YEPD medium containing 0.5% isoamyl alcohol.

Disruption of *GRE2* results in derepressed filamentation

Strain 71.1.2 is disrupted in its *GRE2* gene. Transferring this strain to liquid YEPD medium containing 0.5% isoamyl alcohol resulted in the formation of abnormally long filaments, many of which exceeded 70 μm in length (Fig. 3b). Other strains with the same mutation behaved similarly. The *gre2* mutation caused cells to form long, highly invasive filaments on solid YEPD medium in the absence of isoamyl alcohol (Fig. 4b). The fact that *gre2* mutants formed abnormally long filaments in liquid YEPD containing 0.5% isoamyl alcohol and long, highly invasive filaments in solid YEPD medium in the absence of isoamyl alcohol implies that the *GRE2* gene is a suppressor of filamentation. This idea was confirmed by strain PHY14,

Table 3. The performance of wild-type strain IWD72 and *dld3* mutant strain 70.1.1 in liquid YEPD medium containing various concentrations of isoamyl alcohol

	Percentage of cells having extensions*			
	dld 3		wild type	
Time (h)	0.5% isoamyl alcohol	0.6% isoamyl alcohol	0.5% isoamyl alcohol	0.6% isoamyl alcohol
0	0	0	0	0
2.5	0	0	24.0	16.5
4	0.5	0	53.5	24.5
5.5	3.5	5.0	56.5	33.5
8	10.0	5.5	69.5	53.0
24	3.3	4.5	99.0	88.0

*This includes pseudohyphae, abnormally long buds and all cells whose longitudinal axis was greater than the maximum observed for the same strain growing in liquid YEPD alone. Four hundred cells were counted for each sample.

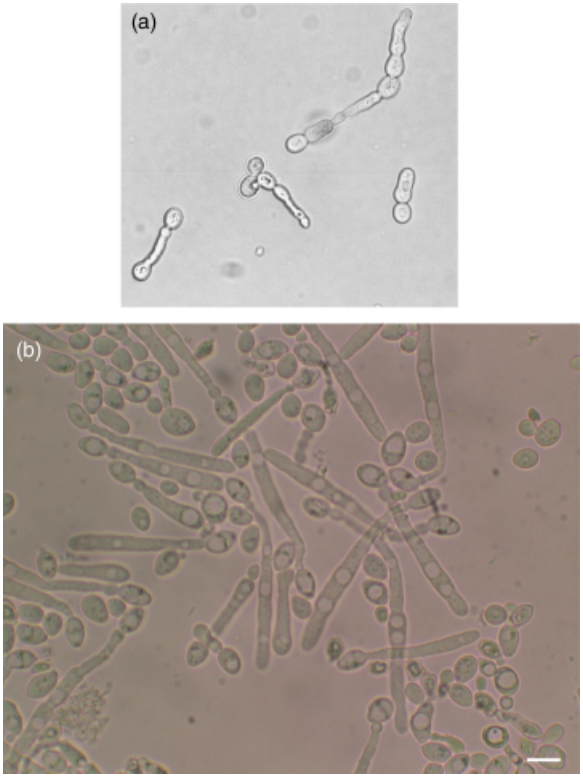

Fig. 3. (a) Wild-type strain IWD72 and (b) *gre2* mutant 71.1.2 in liquid YEPD medium containing 0.5% isoamyl alcohol. The bar marker denotes 10 μm.

which is disrupted in *GRE2* but carries a functional copy of *GRE2* on plasmid pYCG_YOL151W. Strain PHY14 did not form hyperfilaments in liquid YEPD containing 0.5% isoamyl alcohol, and was not hyperinvasive on solid YEPD agar. Instead, when patches of this strain were transferred to solid YEPD medium, the cells only invaded at the edges, leading to the appearance of microcolonies on the surface of the agar (Fig. 4c).

gre2 mutants have reduced activity of isovaleraldehyde reductase

The wild-type strain IWD72 and the *gre2* mutant 71.1.2 were transferred to YEPD medium containing 0.5% isoamyl alcohol and incubated for 5.5 h (the time when methylglyoxal reductase is known to be maximal, as already shown in Fig. 2). The specific activity of methylglyoxal reductase in strain 71.1.2 was about one-third that recorded in the wild type, whereas the specific activity of isovaleraldehyde reductase in the *gre2* mutant was less than half that of the wild type (Table 4). This result is consistent with the idea that *GRE2* encodes isovaleraldehyde reductase activity.

Discussion

In the early stages of isoamyl alcohol-induced filamentation, only four ORFs displayed over 10-fold increases in transcription.

The biochemical, cell biological and morphological consequences of the switch from yeast-form growth to filamentous growth are rapid, profound and dramatic (Kron *et al.*, 1994; Dickinson, 1996, 2004; Rua *et al.*, 2001; Kern *et al.*, 2004), yet despite this, surprisingly few of yeast's *c.* 6100 ORFs displayed very significant increases in transcription of 10-fold or greater. There were only 13 ORFs that showed very significant increases of fivefold or greater (YBL098w, *VAP1*, *LYS20*, *PDR15*, YHR029c, *CUP1A*, *RHR2*, *SNZ1*, *NCE3*, *YGP1*, *PDR16*, YOR049c and *CPA1*). The vast majority, including genes known to be essential to the process, such as *SLT2* (Martinez-Anaya *et al.*, 2003), displayed more modest increases in transcription. The corollary of this is that for the most part, only subtle increases in transcription are required to trigger the switch in growth form. Nevertheless, this study identified two genes (*DLD3* and *GRE2*) as being major contributors at the transcriptional level. Neither had previously been implicated as having roles in filament formation. Both of their respective gene products showed similarly-timed increases in biochemical activity (Figs 1 and 2), indicating that during filamentation, the expression of D-lactate dehydrogenase and methylglyoxal reductase are regulated at the transcriptional level.

DLD3 is required for a complete response to isoamyl alcohol

Previously, no known growth phenotype had been associated with *dld3* mutations (Chelstowska *et al.*, 1999). In the

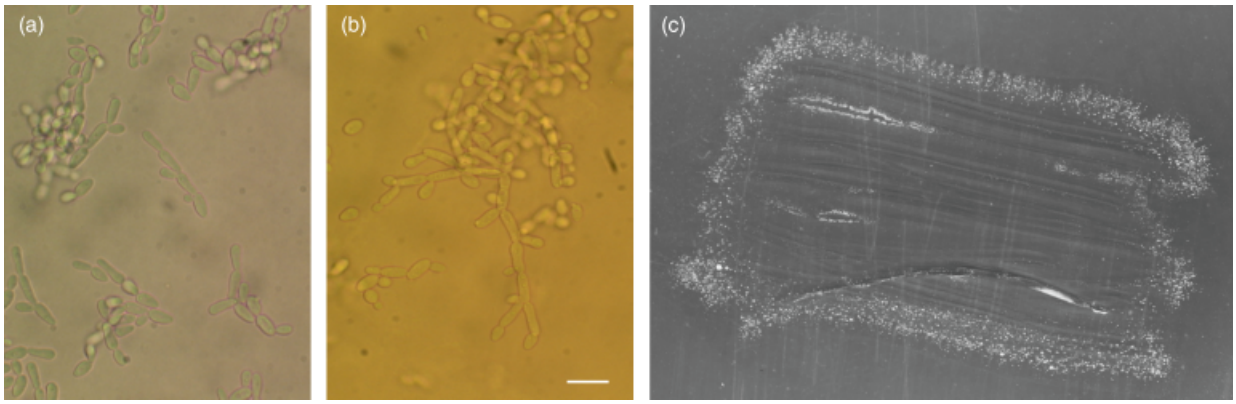

Fig. 4. Invasion of solid YEPD medium by (a) wild-type strain IWD72 and (b) *gre2* mutant 71.5.10. After 2 days of incubation on solid YEPD medium, surface growth was removed by washing with distilled water. Then a slice of the agar was removed aseptically. The top of the agar is at the top of the picture. The bar marker denotes 10 μm. (c) Strain PHY14 (a *gre2* mutant carrying the *GRE2* gene on a plasmid) formed microcolonies when streaked onto solid YEPD medium.

Table 4. The specific activities of methylglyoxal reductase and isovaleraldehyde reductase in extracts from the wild-type and *gre2* mutant strains

Strain	Genetic status	Methylglyoxal reductase (mOD min^{-1} mg^{-1})	Isovaleraldehyde reductase (mOD min^{-1} mg^{-1})
IWD72	Wild type	36.3 ± 6.8 (100%)	11.79 ± 0.1 (100%)
71.1.2	*gre2*	12.3 ± 4.0 (34%)	5.19 ± 0.3 (44%)

The results are the means (± standard error) from two independent cultures for isovaleraldehyde reductase and three independent cultures for methylglyoxal reductase. Each experiment involved duplicate determinations. The activity of the wild-type strain is denoted as 100% in each case.

present study, *dld3* mutants showed reduced levels of filament formation relative to the wild type in the presence of 0.5% isoamyl alcohol, and responded only a little better in 0.6% isoamyl alcohol. This suggests that *DLD3* is required for a full response but is not absolutely essential for it. An alternative explanation is that the gene product of *DLD1* and/or *DLD2* is responsible for the residual low levels of filamentation in the *dld3* mutant strain. It might be argued that such potential redundancy is not really the explanation, because Dld1p and Dld2p are both located in the mitochondrion, whereas Dld3p is a cytosolic enzyme (Lodi & Ferrero, 1993; Chelstowska *et al.*, 1999). However, it is known that isoamyl alcohol-treated cells contain more mitochondrial biomass than untreated cells (Kern *et al.*, 2004), so it seems likely that the mitochondrial enzymes are partially compensating. An unambiguous confirmation of this idea is problematical, because the *dld1 dld2 dld3* triple mutant is nonviable.

GRE2 is a suppressor of filament formation

The *gre2* mutants hyperfilamented (formed unusually long filaments in liquid medium in the presence of 0.5% isoamyl alcohol and produced long, highly invasive filaments on solid YEPD in the absence of isoamyl alcohol) (Figs 3 and 4). This phenotype was reversed by transforming *gre2* mutants with a plasmid carrying the *GRE2* gene. These observations are consistent with the notion that *GRE2* is a suppressor of filament formation in the wild type. Indeed, plasmid-borne *GRE2* appeared to oversuppress to the extent that the growth of transformants on the surface of agar was limited to the formation of microcolonies. This latter observation may be trivial, simply being the consequence of the *GRE2* gene not being in the same context on the plasmid as in its normal chromosomal location.

All of the above requires a biochemical explanation of how the *GRE2* gene product could act during isoamyl alcohol-induced filament formation. One way to reconcile the data is to posit that Gre2p is actually a promiscuous enzyme that will accept isovaleraldehyde (Fig. 5) as a substrate. There is good evidence that this is the case. For example, Rodriguez *et al.* (2001) have demonstrated that Gre2 has α-acetoxy ketone reductase activity and that it reduces α-unsubstituted β-keto esters to (3*S*)-alcohols and most α-substituted β-keto esters to *anti*-(2*S*,3*S*)-alcohols. Hence, although the conventional wisdom held by many yeast researchers is that this enzyme is solely methylglyoxal reductase, it is actually capable of reducing a very wide spectrum of compounds, many of which are much larger molecules than either methylglyoxal or isovaleraldehyde. A closer examination of the literature raises significant doubts about the idea that *GRE2* actually encodes methylgloxal reductase. For example, *gre2*Δ mutants are not sensitive to methylglyoxal (Takatsume *et al.*, 2006.) *GRE3* has been reported to encode an aldose reductase that

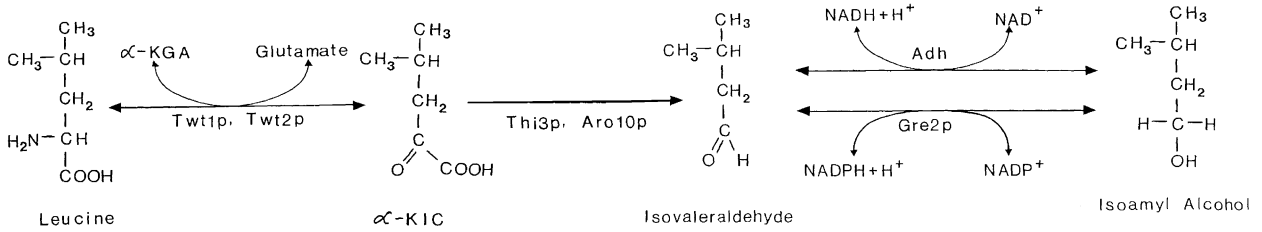

Fig. 5. The pathway of isoamyl alcohol formation in *Saccharomyces cerevisiae*. α-KIC denotes α-ketoisocaproic acid. The enzyme activities identified are: Twt1p and Twt2p – the mitochondrial and cytoplasmic (respectively) branched-chain amino acid aminotransferases; Thi3p and Aro10p – the major and minor (respectively) α-KIC decarboxylases; Gre2p – isovaleraldehyde reductase; Adh – any alcohol dehydrogenase.

is involved in the metabolism of methylglyoxal, but *gre3*Δ mutants are not sensitive to methylglyoxal either (Aguilera & Prieto, 2001). Consequently, the only established system in *S. cerevisiae* for the detoxification of methylglyoxal is via the glyoxalase system (Inoue *et al.*, 1998). This idea is supported by the fact that both *glo1* and *glo2* mutants are sensitive to methylglyoxal (Inoue & Kimura, 1996; Bito *et al.*, 1997).

GRE2 may encode isovaleraldehyde reductase

It seems reasonable to conclude that *GRE2* encodes an isovaleraldehyde reductase activity, because the *gre2* mutant has a much lower level of isovaleraldehyde reductase than the wild-type strain. A total absence of isovaleraldehyde reductase activity in the *gre2* mutant would have been a simpler result to interpret, but our results are reminiscent of those of Ford & Ellis (2002), who noted similar specific activities of methylglyoxal reductase in the wild-type strain and *ypr1* mutants of *S. cerevisiae*, but the activity of 2-methylbutyraldehyde reductase in the *ypr1* mutant was only 48% that in the wild type. These authors and others have acknowledged the difficulty of defining the roles of aldo-keto reductases in many organisms, including yeast due to overlapping substrate specificities and the presence of other related enzymes.

Figure 5 shows the proposed place of Gre2p in relation to the established pathway of isoamyl alcohol formation in *S. cerevisiae* (Dickinson *et al.*, 1997, 2003). It is important to realize that even though the majority of enzyme-catalysed steps within this pathway are reversible, metabolic flux is predominantly in the direction of isoamyl alcohol formation. Hence, when isoamyl alcohol is added exogenously, *GRE2* transcription increases, the activity of Gre2p rises (both shown in this study), and isovaleraldehyde is converted even more quickly to isoamyl alcohol. This consequence is at first sight surprising and seemingly counterintuitive. However, all of the observations can be explained by the notion that the true signal to which cells respond by filamenting is not isoamyl alcohol but isovaleraldehyde. First, the hyperfilamentation of *gre2* mutants in the presence of isoamyl alcohol is explained by the fact that they cannot increase their isovaleraldehyde reductase activity, and hence isovaleraldehyde levels remain higher in these cells. Second, the production of highly invasive filaments even in the absence of isoamyl alcohol can be explained by higher isovaleraldehyde levels in *gre2* mutants, because in the constitutive pathway leading to isoamyl alcohol formation they are not reducing the aldehyde as efficiently as wild-type cells, and so, by virtue of higher isovaleraldehyde levels, are already poised for filament formation. Third, this explains the apparent contradiction that *GRE2* is a suppressor of filament formation whose transcript levels are enhanced by more than 12-fold, and that it encodes an activity that leads to isoamyl alcohol production: increasing Gre2p activity will cause a reduction in isovaleraldehyde (the true signal for filament formation) and hence prevent filamentation. The poor growth and absence of filamentation in the *gre2* mutant carrying plasmid-borne *GRE2* (Fig. 4c) is consistent with the notion that the role of Gre2p is to modulate the extent of filamentous growth.

The current studies do not prove that isovaleraldehyde is the actual signalling compound, but the wild-type strain IWD72 does form filaments in 0.325% (v/v) isovaleraldehyde (Dickinson, unpublished result). (The reader is warned that isovaleraldehyde is volatile and flammable. The necks of culture flasks to which isovaleraldehyde has been added should not be flamed.) Formal confirmation requires additional work that, due to the high volatility of isovaleraldehyde, cannot be performed in the same simple shake flask conditions used here. The two essential but conflicting requirements are to maintain accurate concentrations of isovaleraldehyde in the growth medium for the duration of an experiment (i.e. prevent evaporation) and to keep the cells aerated.

On the day of submission of the revised version of this manuscript, Warringer & Blomberg (2006) published results supporting the possibility that Gre2p is a broad-specificity reductase, mainly acting in ergosterol metabolism with various steroid derivatives as substrates. Their results and ours are not contradictory. Quite clearly, Gre2p has a wide

range of physiological substrates, enabling it to participate in diverse cellular functions.

Acknowledgements

M.H. and P.H. were in receipt of studentships under the European Union's SOCRATES/ERASMUS programme. H.T. was supported by the European Union under the framework of the EUROFAN programme. We thank Susan Budge for technical assistance.

Authors' contributions

M.H and P.H contributed equally to this work.

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