The dihydrosphingosine-1-phosphate phosphatases of *Saccharomyces cerevisiae* are important regulators of cell proliferation and heat stress responses

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We have identified *YSR2* and *YSR3* of *Saccharomyces cerevisiae* as genes encoding dihydrosphingosine-1-phosphate phophatases which are involved in regulation of sphingolipid metabolism [Mao, Wadleigh, Jenkins, Hannun and Obeid (1997) J. Biol. Chem. **272**, 28690–28694]. In this study, we explored the physiological roles that these enzymes may have in *S. cerevisiae*. Deletion of either *YSR2*, *YSR3* or both did not affect viability or growth rate of yeast cells. However, overexpression of YSR2 significantly prolonged the doubling time of cell growth, whereas overexpression of YSR3 affected cell growth only slightly. Cell cycle analysis suggested that overexpression of either YSR2 or, to a lesser extent, YSR3 caused cell cycle arrest at the G1 phase. Disruption of *YSR2*, but not *YSR3*, conferred increased thermotolerance. On the other hand, overexpression of either YSR2 or YSR3 diminished thermotolerance. Using labelled dihydrosphingosine and dihydrosphingosine-1-P (DHS-1-P), we found that overexpression of YSR2 significantly increased ceramide formation, whereas deletion of YSR2, YSR3, or both, accumulated DHS-1-P, and deletion of YSR2 decreased ceramide formation. Together, these results show that the phenotypes of YSR2 are associated with changes in endogenous levels of the different sphingolipids. Green fluorescent protein tagging showed that in the exponentially growing cells, YSR2 and YSR3 had the same cellular localization to endoplasmic reticulum. However, YSR2 and YSR3 differ in mRNA levels: YSR2 had significantly higher mRNA levels than YSR3. This discrepancy might result in the functional differences that these proteins exhibited. In addition, this study implicates sphingolipids and their metabolism in the regulation of growth and heat stress responses of the yeast *S. cerevisiae*.

Key words: sphingolipid metabolism, cell proliferation, sphingosine phosphate phosphatases.

INTRODUCTION

There is ample evidence that metabolites of sphingolipids such as ceramide, sphingosine and sphingosine-1-phosphate (S-1-P) are bio-effector molecules and second messengers in important cellular events, such as cell growth, differentiation, cell senescence, apoptosis and stress responses [1–5]. Both sphingosine and ceramide regulate cell growth [2,5] and apoptosis [3,6]. S-1-P has a proliferative effect on certain cell types [7], and has been shown to trigger intracellular calcium mobilization [8], which is related to stimulation of DNA synthesis. S-1-P also activates phospholipase D, resulting in increases in phosphatidic acid levels [9]. In addition, S-1-P has been implicated in platelet-derived growth factor-mediated signal transduction [10], and recently it has been reported to suppress apoptosis mediated by ceramide [11]. However, regulation of intracellular levels of these signalling molecules is still inadequately understood due to complexity of sphingolipid biosynthesis and metabolism in mammalian cells. The yeast *Saccharomyces cerevisiae* provides a useful system with which to address this issue due to the relative simplicity of its sphingolipids and its susceptibility to genetic manipulation.

The biosynthetic pathway of sphingolipids is conserved from the yeast *S. cerevisiae* to mammalian cells, with some important differences. The yeast *S. cerevisiae* contain dihydrosphingosine (DHS) and phytosphingosine species, but no apparent sphingosine. In mammalian cells, DHS is only found as a precursor to *de novo* synthesis of ceramide, whereas sphingosine is found as a breakdown product of ceramide. In mammalian cells both sphingosine and DHS are phosphorylated to sphingosine (DHS)-1-P by sphingosine kinase [12]. DHS-1-P or S-1-P is then cleaved by S-1-P lyase to ethanolamine phosphate and hexadecenal [13]. Sphingosine (DHS)-1-P is also dephosphorylated to sphingosine (DHS) by S-1-P phosphatase [14].

Evidence for conservation of this metabolic pathway from mammals to yeast was demonstrated recently when the yeast gene for DHS-1-P lyase [16] was identified and cloned. Deletion of *S. cerevisiae* DHS-1-P lyase caused accumulation of DHS-1-P [16]. When this deletion strain was treated with exogenous sphingosine, S-1-P accumulated and appeared to be toxic to the yeast cells. We recently demonstrated that this toxicity could be rescued by overexpression of the yeast gene *YSR2*, which is what led to its cloning and identification as a DHS-1-P phosphatase [15]. We also identified another DHS-1-P phosphatase, YSR3, based on 53% identity at the protein level. Both proteins have *in vitro* activity of DHS-1-P phosphatase. The former of these proteins, YSR2, appeared to be involved in regulating uptake and metabolism of DHS in *S. cerevisiae* [15]. Deletion of *YSR2*

Abbreviations used: CAPP, ceramide-activated protein phosphatase; GFP, green fluorescent protein; S-1-P, sphingosine 1-phosphate; DHS, dihydrosphingosine; DHS-1-P, dihydrosphingosine 1-phosphate; SL, sphingolipid; IPC, inositol-phosphorylceramide; MIPC, mannose-inositol-phosphorylceramide; $M(IP)_2C$, mannose-(inositol-phosphoryl)$_2$-ceramide; PKC, protein kinase C; RT-PCR, reverse transcriptase PCR; PI, phosphatidylinositol; SC, synthetic minimal medium; SC−ura, SC without uracil; *YSR3* is used in this study instead of *YSR2-1* in the previous paper [15] to agree with the guidelines of naming *Saccharomyces cerevisiae* genes.

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significantly reduced exogenous DHS incorporation into sphingolipids and caused a decrease in endogenous sphingolipid synthesis of *S. cerevisiae*. *YSR2* was also independently identified as a gene whose deletion was associated with accumulation of long-chain bases [17].

The biological role of these enzymes in *S. cerevisiae* remains unclear. In order to evaluate that role, we created yeast strains that harbour deletions or overexpression of one or both of these genes. In this study we demonstrate that the deletion of both phosphatase genes caused accumulation of DHS-1-P, but did not affect cell growth. However, overexpression of one of these proteins, YSR2, significantly suppressed yeast cell growth. We show that this is due to accumulation of ceramide and inositol-ceramide sphingolipids. We also demonstrate that the DHS-1-P phosphatase (YSR2) has an important role in heat stress responses. The results also show a difference in the two DHS-1-P phosphatase genes (*YSR2* and *YSR3*). Using reverse transcriptase PCR (RT-PCR), we demonstrate that YSR2 and YSR3 genes are transcribed at different levels. This difference may account for the different functions exhibited by YSR2 and YSR3. These results implicate ceramide and other sphingolipids in modulating *S. cerevisiae* stress responses.

EXPERIMENTAL

Strains and media

The yeast strains listed in Table 1 were maintained on YPD medium [1 % (w/v) yeast extract/2 % (w/v) peptone/2 % (w/v) glucose] or synthetic minimal medium (SC) with or without (SC − ura) uracil as described [15]. To determine growth effects associated with disruption of *YSR2*, *YSR3*, or both, a single colony of the different strains was inoculated into 5 ml of YPD medium and incubated at 30 °C. Overnight cultures or exponentially growing cells were diluted with fresh medium to a density of D_{600} 0.1–0.2 and incubated at 30 °C. Growth curves were determined by measurement of D_{600} at different time points. To determine the effect of overexpression of YSR2 or YSR3 on growth, cells were cultured in SC − ura medium plus 2 % glucose or galactose at 30 °C.

Flow cytometry analysis

Cells were harvested at different phases of growth, washed once with water, and fixed in 70 % ethanol at 4 °C. FACS analysis was performed with a fluorescence-activated cell analyser (Becton Dickinson) as described [18].

Heat tolerance

Cells from exponentially growing cultures were diluted with water to a density of 3×10^6 cells/ml and incubated at 50 °C or 55 °C for different time periods. The same number of cells, before and after heat shock treatment, were plated onto YPD medium. The viability after heat shock was calculated by comparison of colony numbers on the plates before and after heat shock.

Lipid analysis

Yeast cells were labelled with either D-*erythro*-[4,5-^{3}H]DHS, D-*erythro*-[4,5-^{3}H]DHS-1-P or [^{3}H]inositol, and radiolabelled lipids were extracted and resolved on Silica gel 60 TLC plates as described [15]. In order to better resolve sphingolipids by TLC, glycerolipids were degraded and removed by base (monomethlylamine) hydrolysis as described [19]. Labelled lipids were detected and quantified by a PhosphorImager and ImageQuant software (Storm, Molecular Dynamics) as recommended by the manufacturer. Ceramide measurement was performed by the diacylglycerol kinase method [15].

Yeast gene disruption

The primers (#1 and #2) for constructing the disruption cassettes are listed in Table 2. The G418 resistance gene in Δ*ysr3* was replaced with the *URA3* gene by transforming the Δ*ysr3* mutant cells with the disruption cassette containing the *URA3* gene flanked by portions of sequence of *YSR3* as described [15]. The *YSR3* deletion mutant Δ*ysr3* with *URA3* as the marker was selected on SC − ura plates and by loss of G418 resistance. This mutant (Δ*ysr3-1*) was crossed with the Δ*ysr2*, the resultant diploid cells were selected on SC − ura medium containing 225 μg/ml G418, and were sporulated on the sporulation medium YEPA (1 % yeast extract/2 % peptone/2 % potassium acetate) [20]. The tetrads were dissected as described [15]. The haploid strain (Δ*ysr2*Δ*ysr3-1*) with double-deletion of *YSR2* and *YSR3* was identified as G418 resistant and prototrophic for uracil, and was confirmed by PCR. The *URA3* gene, when used as a selectable marker in the Δ*ysr2*Δ*ysr3-1* mutant, interfered with some phenotypes. It was therefore replaced with the G418 resistance gene to create a new double-deletion mutant (Δ*ysr2*-Δ*ysr3*) without the *URA3* gene. The *URA3* gene replacement was selected by 5-fluoro-orotic acid medium in which only the *URA3*-negative clones could survive.

Yeast gene expression

The yeast genes *YSR2* and *YSR3* were tagged with the Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys (FLAG) peptide coding sequence and were subcloned into the vector pYES2 as described [15]. The vector (pYES2) and the constructs expressing *YSR2* (pYSR2) or *YSR3* (pYSR3) were respectively introduced into the wild-type (JK9-3d) cells as described [15]. The coding sequence of GFPuv was amplified from the plasmid pGFPuv by PCR using the primers (#3) listed in Table 2, and was cloned into the *Hin*dIII

Table 1 Yeast strains used in this study

Strain	Genotype	Phenotype	Source
JK9-3d α	*MATα leu2-3,112 ura3-52 rme1 trp1 his4 HMLa*	Wild type, YSR2$^+$, YSR3$^+$	[15]
JK9-3d a	*MATa leu2-3,112 ura3-52 rme1 trp1 his4 HMLa*	Wild type, YSR2$^+$, YSR3$^+$	[15]
JK9-3d-*URA3*	*MATa leu2-3,112 ura3-52::URA3 rme1 trp1 his4 HMLa*	*URA3* $^+$	This study
Δ*ysr2*	*MATα leu2-3,112 ura3-52 rme1 trp1 his4 HMLa ysr2*Δ*::NEO*	*YSR2* deletion	[15]
Δ*ysr3*	*MATα leu2-3,112 ura3-52 rme1 trp1 his4 HMLa ysr3*Δ*::NEO*	*YSR3* deletion	[15]
Δ*ysr3-1*	*MATa leu2-3,112 ura3-52 rme1 trp1 his4 HMLa ysr3*Δ*::URA3*	*YSR3* deletion	This study
Δ*ysr2*Δ*ysr3*	*MATα leu2-3,112 ura3-52 rme1 trp1 his4 HMLa ysr2*Δ*::NEO ysr3*Δ*::NEO*	*YSR2* and *YSR3* deletions	This study

Table 2 Primers used for PCR in this study

Primer pair		Oligonucleotide sequence	Purpose
1#	Forward	5′ TCAGACGGTTACTGAATTGGGTGTTACCGAGGACACCGTCCGTG GAATTCTCATGTTTGACAGCTTATCATC G 3′	Creating Δ*ysr3-1*
	Reverse	5′ GGTTGTAGGTATACCTGCGTATAGAGAAACCTTCCGACGGCATC CAGGGTGACGGTGCCGAGGATGACGATGAGCG 3′	
2#	Forward	5′ CAGCTGAAGCTTCGTACGCTGCAGGTCGACGGATCCCGTCCGTGG AATTCTCATGTTTGACAGCTTATCATCG 3′	Creating Δ*ysr2*Δ*ysr3*
	Reverse	5′ GCATAGGCCACTAGTGGATCTGATATCATCGATGAATTCGGCATC CAGGGTGACGGTGCCGAGGATGACGATGAGCG 3′	
3#	Forward	5′ CCCAAGCTTATGGCTAGCAAAGGAGAAGAAC 3′	Subcloning GFPuv
	Reverse	5′ CCCGGTACCTTTGTAGAGCTCATCCATGCC 3′	
4#	Forward	5′ CGG GGT ACC ATG GGATATTTTGTTCCGGATTCAC 3′	Subcloning EPT1
	Reverse	5′ CGGGAATTCTTATGTCAGCTTGGAGCGCTTGATGG 3′	
5#	Forward	5′ ATGGATTCTGGTATGTTCTA3′	RT-PCR of actin
	Reverse	5′ GATACCTCTCTTGGATTGAGC3′	
6#	Forward	5′ ATGGTAGATGGACTGAATACCTCG3′	RT-PCR of YSR2
	Reverse	5′ TATTTAAGAGGGAAAATAGGACGGG3′	
7#	Forward	5′ ATTATTCAGACGGTTACTGAATTGGG3′	RT-PCR of YSR3
	Reverse	5′ TCCAAGTGAAAAAAACTGGGCATAGC3′	

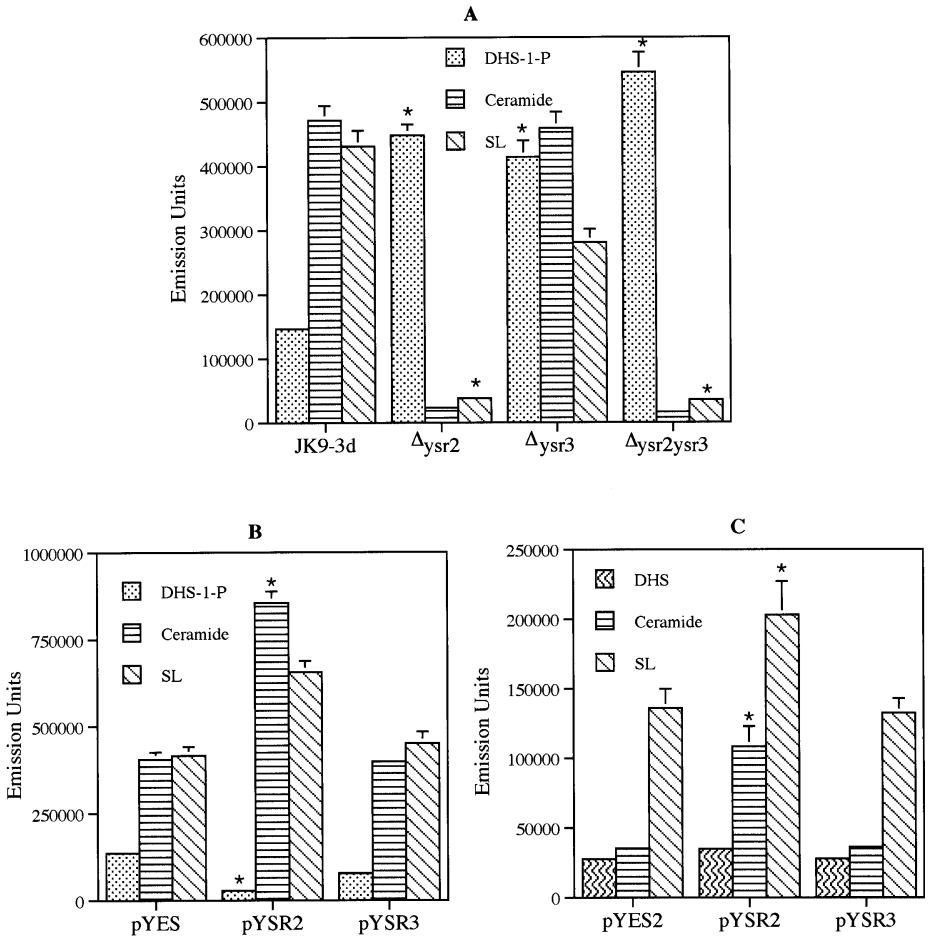


Figure 1 Control of flux of sphingoid bases by DHS-1-P phosphatases YSR2 and YSR3 in metabolism and biosynthesis of sphingolipids

The double-deletion mutant (Δ*ysr2*Δ*ysr3*) bearing the *ysr2* and the *ysr3* deletion alleles was created as described in the Experimental section. The cells of the deletion mutants (Δ*ysr2*, Δ*ysr3* or Δ*ysr2*Δ*ysr3*) were labelled with [^{3}H]DHS (**A**) and the cells overexpressing YSR2 or YSR3 were labelled with [^{3}H]DHS (**B**) or [^{3}H]DHS-1-P (**C**) as described in the Experimental section. Total lipids were extracted, separated on TLC, scanned by the PhosphorImager and quantified by ImageQuant software (Molecular Dynamics). Emission units, which were given by the ImageQuant, represent the relative intensity of the radioactive lipid bands. The results represent means ± S.D. of two separate experiments. $^*P < 0.05$ based on paired Student's *t*-test. SL, sphingolipids.

and *Kpn*I sites of the plasmids pYSR2 and pYSR3. [GFP is the green fluorescent protein of *Aequorea victoria* and GFPuv is a GFP variant (Clontech, Inc.) with excitation in the uv region.] The GFP sequence placed in frame with the 5′-end of the coding sequence of the YSR2 or YSR3 on the plasmid pYSR2 or pYSR3 was confirmed by DNA sequencing. The coding sequence of *sn*-1,2-diacylglycerol ethanolamine- and choline-phosphotransferase (EPT1) as a marker protein for endoplasmic reticulum was amplified by PCR from *S. cerevisiae* genomic DNA using #4 primers listed in Table 2. The coding sequence of EPT1was inserted into the *Kpn*I and *Eco*RI sites of the plasmid pYES2-GFPuv which was constructed by inserting the GFPuv coding sequence into the vector pYES2. The constructs expressing GFPuv or the GFPuv fusion proteins YSR2–GFPuv, YSR3–GFPuv and EPT1–GFPuv were introduced into the JK9-3d cells. Gene expression was induced by incubating cells in SC − ura medium plus 2% galactose.

Confocal microscopy of the GFPuv-tagged proteins

The cells expressing GFPuv or GFPuv fusion proteins were collected by centrifugation at 950 ***g*** for 5 min and washed with PBS once. The cells were resuspended in PBS at a density of 10^9 cells/ml, and 3 μl of cells was spotted onto a glass slide onto which a cover slide was mounted and sealed with the mounting medium Cytoseal 60 (Stephens Scientific). Fluorescence of cells was examined under a confocal system (Olympus, LSM-TSU).

Western blotting analysis of the tagged proteins

Extraction of total protein from yeast cells, determination of protein concentrations, SDS/PAGE, protein transfer and Western blotting analysis were carried out as described [15]. The fusion proteins YSR2–GFPuv and YSR3–GFPuv were detected by the GFP monoclonal antibody (1:1000; Clontech) against GFP using an ECL plus kit (Amersham).

Quantification of mRNA by RT-PCR

Total RNA was extracted from yeast cells. DNase I (Promega) was added to the RNA samples to digest DNA which would interfere with RT-PCR. RT-PCR was performed using a kit (Gibco BRL) as recommended by the manufacturer. The primers for RT-PCR are listed in Table 2.

RESULTS

Deletion or overexpression of YSR2 or YSR3 alters metabolism of sphingolipids in *S. cerevisiae*

YSR2 and *YSR3* are two *S. cerevisiae* genes that encode for DHS-1-phosphate phosphatases which dephosphorylate DHS-1-P [15]. In characterizing these genes and their physiological roles in *S. cerevisiae*, we previously demonstrated that deletion of either *YSR2* or *YSR3* resulted in accumulation of DHS-1-P. We also demonstrated that deletion of *YSR2* eliminated biosynthesis of sphingolipids, including dihydroceramide, from exogenous DHS [15]. Deletion of either phosphatase gene did not appear to affect viability or growth of yeast cells.

In this study, we elected to delete both *YSR2* and *YSR3* to eliminate the possibility that the phenotypes caused by deletion of one gene might be compensated for by the other. Upon deleting both genes, we found that the mutant (Δ*ysr2*Δ*ysr3*) bearing both the *YSR2* deletion allele and the *YSR3* deletion allele accumulated more DHS-1-P than either of the single-deletion mutants (Δ*ysr2* and Δ*ysr3*), as determined by labelling with [^{3}H]DHS (Figure 1A). In addition, the Δ*ysr2* or the Δ*ysr2*Δ*ysr3* mutant had reduced DHS incorporation into ceramide, and all deletion mutants had reduced DHS incorporation into sphingolipids (Figure 1A). These results suggested that YSR2, and to a lesser extent YSR3, were required for biosynthesis of sphingolipids from exogenous DHS.

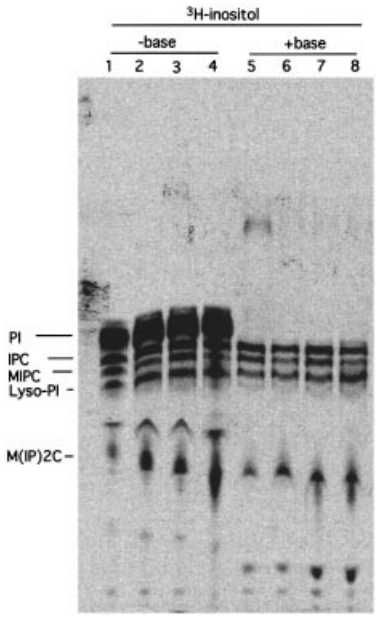


Figure 2 Reduction of endogenous sphingolipid biosynthesis by double-deletion of *YSR2* and *YSR3*

The wild-type cells, the double-deletion mutant cells and single-mutant cells were labelled with [^{3}H]inositol. Total lipids were subjected to base (monomethylamine) hydrolysis (+ base, lanes 1–4), or were not (− base, lanes 5–8), as described in the Experimental section. Lipids were separated on TLC and analysed by the PhosphorImager as described in the Experimental section. Lanes 1 and 5, wild-type strain (JK9-3d α); lanes 2 and 6, *YSR2* deletion mutant (Δ*ysr2*); lanes 3 and 7, *YSR3* deletion mutant (Δ*ysr3*); lanes 4 and 8, double-deletion mutant (Δ*ysr2*Δ*ysr3*).

In order to evaluate *de novo* synthesis of sphingolipids by these mutants, we next labelled the mutants with [^{3}H]inositol. Major sphingolipids such as inositol-phosphorylceramide (IPC), mannose IPC (MIPC) and mannose-(inositol-phosphoryl)$_2$-ceramide [M(IP)$_2$C] were still synthesized, but the *YSR2* deletion mutant (Δ*ysr2*) and the double-deletion mutant (Δ*ysr2*Δ*ysr3*) had somewhat less sphingolipids than the wild-type strain (Figure 2).

Interestingly, the single mutants (Δ*ysr2* and Δ*ysr3*) and the double-deletion mutant (Δ*ysr2*Δ*ysr3*) had higher levels of phosphatidylinositol (PI) when labelled with [^{3}H]inositol (Figure 2). In previous work, we demonstrated that deletion of the *S. cerevisiae* DHS-1-P lyase (encoded by BST1) accumulated DHS-1-P and blocked biosynthesis of glycerolipids, including PI, from the pathway of DHS to DHS-1-P. In contrast, deletion of YSR2 increased incorporation of DHS into glycerolipids such as phosphatidylcholine, phosphatidylethanolmine and PI [15]. DHS-1-P phosphatase (YSR2) and DHS-1-P lyase have the same substrate, DHS-1-P, but have different products. Therefore YSR2 and BST1 have an opposite role in controlling the flux of phosphorylated sphingoid bases between sphingolipids and glycerolipids. YSR2 appears especially critical for incorporation of the phosphorylated DHS into sphingolipids. Taken together, these results suggest that exogenous, and possibly endogenous, DHS is phosphorylated and dephosphorylated before incor-

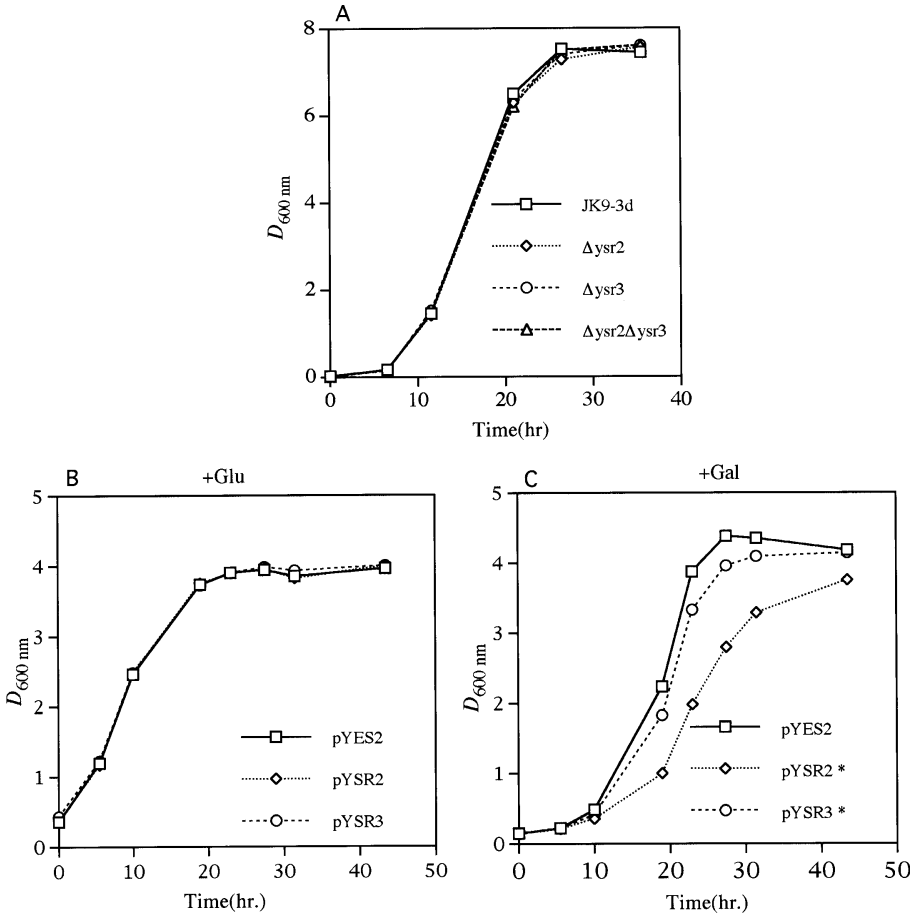


Figure 3 Effect of overexpression of *YSR2* and *YSR3* on cell growth of the yeast *S. cerevisiae*

The cells of the mutant strains Δ*ysr2*, Δ*ysr3* and Δ*ysr2*Δ*ysr3* were cultured in YPD medium, whereas the cells overexpressing *YSR2* and *YSR3* were cultured in SC—ura medium plus glucose or galactose as described in the Experimental section. (**A**) Growth curves of the mutants (Δ*ysr2*, Δ*ysr3* and Δ*ysr2*Δ*ysr3*) and their wild type (JK9-3d α); (**B**) growth curves of overexpression strains (pYSR2 and pYSR3) and the vector control strain (pYES2) in SC—ura medium plus glucose; (**C**) growth curves of the same strains as in (**B**) in galactose medium. The *y*-axis represents the attenuance at 600 nm. The results represent means ± S.D. of three independent experiments. The error bars are too small to be seen. $^*P < 0.05$ based on the paired Student's *t*-test.

poration into sphingolipids. YSR2 is mostly responsible for this dephosphorylation.

On the other hand, we speculated that overexpression of DHS-1-P phosphatases might decrease DHS-1-P levels, thus increasing DHS levels and causing more ceramide and sphingolipids synthesis. We next overexpressed *YSR2* or *YSR3* under the control of the Gal1 promotor. Upon [^{3}H]DHS or [^{3}H]DHS-1-P labelling, cells overexpressing YSR2 had lower levels of DHS-1-P and indeed had higher levels of ceramide and other sphingolipids synthesized from DHS (Figure 1B) or DHS-1-P (Figure 1C). Overexpression of *YSR3* did not significantly change levels of ceramide and other sphingolipids, although DHS-1-P levels decreased compared with wild-type cells (Figure 1B). To investigate whether changes in sphingolipid labelling mirror changes in mass of sphingolipids in the cells, we measured the concentration of ceramide using the diacylglycerol kinase method. The YSR2-expressing cells had a 2-fold higher concentration of ceramide (120.0 ± 26.2 pmol ceramide/μmol P_i) compared with the wild-type cells which contained 63.0 ± 19.0 pmol ceramide/μmol P_i and the YSR3-expressing cells which contained 56.0 ± 22.4 pmol ceramide/μmol P_i. These results suggest that YSR2 is the predominant phosphatase required for balancing levels of various sphingolipids in yeast cells.

Cell growth is affected by overexpression of YSR2

Ceramide and sphingosine have been demonstrated to be involved in mediating signals which suppress cell proliferation in mammalian cells and yeast cells, whereas S-1-P has a proliferative effect. However, although deletion of YSR2, YSR3 or both caused an increase in DHS-1-P levels in cells, cell growth was not affected by this increase (Figure 3A). We next addressed the question of whether an increase in ceramide levels caused by overexpression of YSR2 suppresses cell proliferation. When the cells were grown in the presence of glucose, which suppressed gene expression under the control of the Gal1 promotor, the transformed genes were not expressed and there was no difference in growth among all three strains (Figure 3B). However, when the cells were grown in the presence of galactose, which induced overexpression of the genes, overexpression of YSR2 significantly prolonged yeast doubling time, and overexpression of YSR3 slightly affected this process (Figure 3C).

To investigate whether *YSR2* and *YSR3* control cell growth

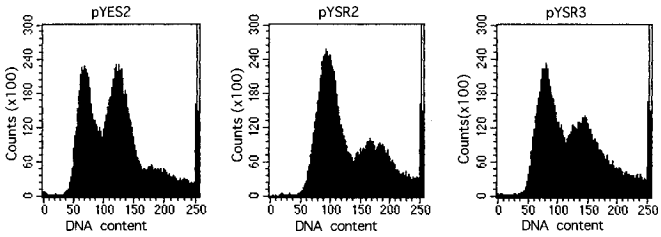


Figure 4 Arrest of cell cycle of *S. cerevisiae* at G1 phase by overexpression of YSR2

Cells were collected after 14 h of growth, fixed by 70% ethanol and monitored by FACS analysis as described in the Experimental section. FACS histograms of the cells were obtained with the vector(pYES2) only and the cells overexpressing *YSR2* or *YSR3*. *x*-axis, DNA content; *y*-axis, cell numbers.

through regulation of the cell cycle, we next analysed the cell cycle phenotype of the cells overexpessing *YSR2* and *YSR3*. Using flow cytometry studies, we found that overexpression of YSR2, and to a lesser extent YSR3, arrested cells prematurely at the G_1 phase of the cell cycle (Figure 4). Moreover, when the wild-type cells transformed with the vector pYES 2 entered the exponential phase, more than 80% of the cells formed buds, whereas less than 25% of the YSR2-overexpressing cells budded. Overexpression of YSR3 affected cell budding slightly, whereby 70% of the cells formed buds. In addition, whereas the wild-type cells entered the stationary phase, most of the cells with overexpression of YSR2 appeared in doublets in which the daughter cells did not separate from the mother cells until they reached the same size as the mother cells. However, the YSR3-overexpressing cells behaved similarly to the wild type in this phase. Together, these results suggest that ceramide, whose levels are elevated by overexpression of YSR2, has a role in inhibiting growth.

Disruption of *YSR2* confers a thermotolerant phenotype

Previous studies have demonstrated that sphingolipids have an important role in stress responses, specifically in heat stress [21,22]. When yeast cells were heat shocked at 39 °C, both phytosphingosine and ceramide levels were shown to increase [21,22]. To examine whether alteration of sphingolipid metabolism by disruption of *YSR2*, *YSR3*, or both *YSR2* and *YSR3*, regulated thermotolerance, the cells of the wild-type strain and the single-deletion or double-deletion mutants were treated with heat shock at 50 °C for 40 min or 55 °C for 7 min. The viabilities of different strains subjected to heat stress were compared. Table 3 demonstrates that the viability of the *YSR2* deletion mutant was more than 3-fold higher than that of the wild type. The viability of the *YSR3* deletion mutant was the same as that of the wild type. The double-deletion mutant (Δ*ysr2*Δ*ysr3*) exhibited the same increased level of thermotolerance as the Δ*ysr2* mutant. These results suggest that alterations in the metabolism of sphingolipids may play a role in heat stress responses.

Since overexpression of YSR2 increased levels of ceramide and other sphingolipids, we predicted that overexpression of YSR2 would sensitize cells to heat stress responses. To investigate this, cells overexpressing YSR2 were treated as described above. We found that overexpression of YSR2 did impart heat sensitivity to cells (Table 3). On the other hand, overexpression of YSR3 also sensitized cells to heat stress (Table 3), even though it did not change the levels of sphingolipids, except for decreasing DHS-1-P levels. Therefore the sensitivity of heat stress may be attributable to both a decrease in DHS-1-P and increases in other sphingolipids.

Table 3 Deletion of *YSR2* conferred thermotolerance

The cells of the mutants (Δ*ysr2*, Δ*ysr3* and Δ*ysr2*Δ*ysr3*) and the wild type (JK9-3d α) were grown in YPD medium. The cells overexpressing YSR2 or YSR3 and the cells transformed with the empty vector (pYES2) were grown in SC-ura medium with 2% galactose. For heat shock, the exponentially growing cells of the different strains were incubated at 50 °C for 40 min or 55 °C for 7 min; the cells before and after heat shock were plated on YPD plates and incubated at 30 °C for 2–3 days. Viability was determined as described in the Experimental section. Numbers in the Table represent post-heat-shock viability of the wild type cells (JK9-3d α), the mutant cells (Δ*ysr2*, Δ*ysr3* and Δ*ysr2*Δ*ysr3*), the cells with the vector (pYES2) only and the cells overexpressing *YSR2* or *YSR3*, and viability of the *URA3* minus cells (JK9-3d a) and the *URA3* plus cells (JK9-3d-*URA3*). Results represent means ± S.E.M of three independent experiments, where the values are percentage of cells surviving after heat treatment. $^*P < 0.05$ based on paired Student's *t*-test. For each set of data, its wild-type or vector-transformed strain was used as a control. N/T, not tested.

Strain	50 °C 40 min	55 °C 7 min
JK9-3d	1.1 ± 0.4	2.5 ± 0.45
Δ*ysr2*	6.3 ± 1.2*	6.4 ± 1.1*
Δ*ysr3*	1.2 ± 0.4	3.4 ± 0.6
Δ*ysr2*Δ*ysr3*	6.8 ± 1.7*	7.0 ± 2.1*
pYES2	6.9 ± 2.0	9.6 ± 1.9
pYSR2	0.1 ± 0.0*	0.6 ± 0.2*
pYSR3	3.4 ± 1.0	3.8 ± 0.6*
JK9-3d a	1.4 ± 0.1	N/T
JK9-3d-U a	26.5 ± 0.4*	N/T

Cellular localization of YSR2 and YSR3 is correlated with their functions as regulators of sphingolipid biosynthesis and metabolism

Although both YSR2 and YSR3 proteins have *in vitro* activity of DHS-1-P phosphatase, when studied in cells they do not appear to have the same role in regulation of the metabolism of DHS and DHS-1-P and in the regulation of cell growth and heat stress responses. Deletion of YSR2 blocks incorporation of exogenous DHS into sphingolipids, resulting in increases in glycerolipids. In contrast, overexpression of YSR2 reverses DHS flux between sphingolipids and glycerolipids. Deletion or overexpression of YSR3 does not appear to affect DHS metabolism to the same extent. To investigate whether the difference in regulation of DHS metabolism by YSR2 and YSR3 is due to a difference in localization, we tagged the YSR2 and the YSR3 protein with GFP. Figure 5 demonstrates that the fusion proteins YSR2–GFPuv and YSR3–GFPuv in the exponentially growing cells had the same cellular localization. In agreement with the fluorescent pattern of another endoplasmic reticulum protein *sn*-1,2-diacylglycerol ethanolamine- and choline-phosphotransferase (encoded by EPT1), they seem to be predominantly localized to the endoplasmic reticulum, where biosynthesis of most sphingolipids occurs. These results suggest that YSR2 and YSR3 have the same cellular localization, and the functional differences between YSR2 and YSR3 cannot be attributed to their cellular localization.

YSR2 and YSR3 have different transcription levels

We next evaluated the gene expression of YSR2 and YSR3. Messenger RNA levels of YSR2 and YSR3 were quantified using

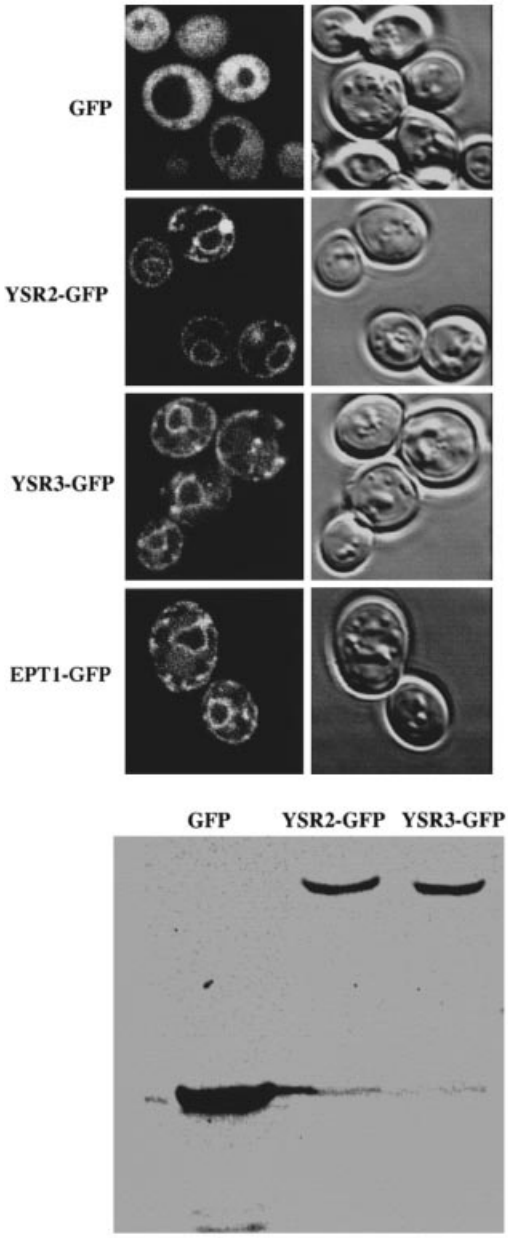


Figure 5 Localization of YSR2 and YSR3 to endoplasmic reticulum

Constructs expressing the fusion proteins YSR2–GFPuv and YSR3–GFPuv as well as GFPuv–EPT1, the marker protein for endoplasmic reticulum, were introduced into the JK9-3d cells as described in the Experimental section. Cells expressing the GFPuv fused proteins were cultured in SC − ura medium plus 2% galactose for 10 h. Fluorescence of GFP or GFP fusion proteins in the cells was visualized under the confocal system. The cells at the corresponding time point were harvested and subjected to protein extraction for Western blotting analysis as described in the Experimental section. Top: confocal images; bottom: Western blot.

RT-PCR. The primers used for RT-PCR of YSR2 and YSR3 were the same ones used for subcloning their open reading frames, which were confirmed by DNA sequencing. Therefore, the RT-PCR products are specific. YSR2 had significantly higher (4-fold, quantified using the ImageQuant) mRNA levels than YSR3 (Figure 6). These results suggest that YSR2 is expressed at significantly higher levels and, in most cases, this is reflected in higher amounts of protein. This lends credence to the different

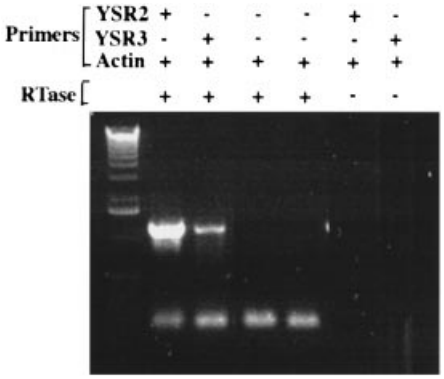


Figure 6 Different transcription levels of YSR2 and YSR3

Total RNA was extracted from the exponentially growing cells (JK9-3d α) as described in the Experimental section. Total RNA (4 μg) was reverse transcribed and followed by PCR using different specific primers as indicated. The yeast actin gene was used as an internal standard in RT-PCR of YSR2 or YSR3. The RT-PCR products were run on a 1% agarose gel in the presence of ethidium bromide. The gel was photographed and scanned by a Storm Imaging system (Molecular Dynamics Inc.). The density of the DNA bands was determined by the ImageQuant software.

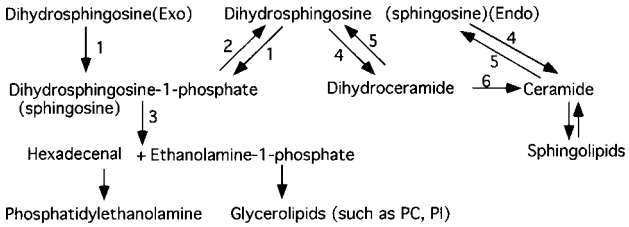
roles these proteins have in sphingolipid metabolism and stress responses in *S. cerevisiae*.

DISCUSSION

In this study, we investigated the metabolic and physiological roles of the *S. cerevisiae* DHS-1-P phosphatases YSR2 and YSR3. We demonstrate that deletion of both YSR2 and YSR3 causes yeast cells to accumulate more DHS-1-P than single deletion of either *YSR2* or *YSR3*. Similar to the *YSR2* deletion mutant, the double-deletion mutant cannot incorporate exogenous DHS into sphingolipids. On the other hand, overexpression of YSR2 decreases cellular DHS-1-P levels and increases ceramide levels. Overexpression of YSR2 also leads to suppression of cell growth, suggesting that the accumulated ceramide is important in inhibition of cell growth and that this pathway is conserved from mammalian to yeast cells. We also demonstrate that *YSR2* deletion confers thermotolerance and that overexpression of either YSR2 or YSR3 decreased thermotolerance. We demonstrate that YSR2 has significantly higher mRNA levels than YSR3, thus explaining the potential differences in function attributed to these proteins.

Ceramide and sphingosine have been shown to cause growth arrest in both mammalian [3] and *S. cerevisiae* cells [23,24]. On the other hand, S-1-P has been shown to stimulate cell proliferation in mammalian cells [7]. Therefore, levels of ceramide, DHS (or phytosphingosine) and phosphorylated sphingoid bases may be critical for determining whether the yeast *S. cerevisiae* cells proliferate or undergo growth arrest. Regulation of the enzymes involved in metabolism of these lipid molecules has become an important area of study. We and other groups have identified and characterized several such enzymes which are involved in the biosynthesis and metabolism of ceramide, DHS and its phosphorylated form DHS-1-P. The identification of these enzymes should facilitate the study of the metabolism and physiological functions of sphingolipids.

In *S. cerevisiae*, DHS can be either acylated by ceramide



Dihydrosphingosine(sphingosine) metabolism

1. Dihydrosphingosine(sphingosine) kinase, 2. Dihydrosphingosine phosphatases (*YSR2/LCB3/LBP1*), 3. Dihydrosphingosine lyase(*BST1*), 4. Ceramide synthetase, 5. Ceramidase, 6. Dihydroceramide desaturase

Exo: exogenous, Endo: endogenous.

Scheme 1 Metabolism of sphingoid bases in the yeast *S. cerevisiae*

synthetase or phosphorylated by DHS kinase (Scheme 1). If acylated, DHS forms ceramide and enters the biosynthetic pathway. If phosphorylated, DHS is converted into DHS-1-P and enters the catabolic pathway. Phosphorylated DHS has two fates: on the one hand, it is cleaved by DHS-1-P lyase to ethanolamine phosphate and hexadecenal, which are incorporated into glycerolipids [15,16]; on the other hand it is dephosphorylated by DHS-1-P phosphatase to DHS which is then acylated to ceramide and re-enters the biosynthetic pathway [15]. As demonstrated in the present paper, overexpression of YSR2 significantly enhanced this biosynthetic pathway, thus increasing the levels of ceramide and IPCs but decreasing the levels of DHS-1-P in cells (Figures 1B and 1C). On the other hand, overexpression of *YSR3* did not appear to cause significant increases in DHS or ceramide, but did result in a slight (but significant) decrease in DHS-1-P. Increases in ceramide and a decrease in DHS-1-P may be synergistically responsible for the growth inhibition, which may explain why overexpression of YSR2 inhibited cell growth more significantly than that of YSR3.

Changes in cellular levels of the different sphingolipids alter the stress responses of *S. cerevisiae* cells and affect cell growth. In rich medium with fermentable sugar such as glucose as a carbon source, yeast grows anaerobically and rapidly. When the fermentable sugar is exhausted and ethanol is produced, yeast cells use ethanol as a carbon source for slower aerobic growth. The switch from anaerobic growth to aerobic growth with depletion of glucose is called the diauxic shift. It has been demonstrated [25] that deletion of the DHS-1-P lyase accumulates DHS-1-P and leads to faster post-diauxic growth. On the other hand, we demonstrate that an increase in ceramide formation by overexpression of YSR2 caused inhibition of cell growth. These results suggest that DHS-1-P promotes cell growth whereas ceramide suppresses cell growth. It is also possible that other metabolites of these molecules are responsible for the observed effects.

The mechanism by which these lipids regulate cell growth is not well understood. Ceramide is an inhibitor of protein kinase C (PKC) [26], and is known to activate a protein phosphatase termed ceramide-activated protein phosphatase (CAPP) [23]. In the yeast *S. cerevisiae*, both PKC1 [27] and a protein phosphatase PP2A [28] (which belongs to the same phosphatase class as CAPP) have been implicated in the regulation of cell proliferation. PKC1 is required for yeast viability whereas PP2A has been demonstrated to regulate cell cycle progression. The increase in ceramide levels that we see upon overexpression of YSR2 may lead to inhibition of PKC1 and to activation of CAPP, thus leading to the observed cell cycle arrest. Further studies are required to elucidate the mechanism involved.

Another important observation from this study and other studies involves the role of these sphingolipids in thermotolerance. Dickson et al. [21] and Jenkins et al. [22] have shown that DHS and ceramide increase significantly upon heat shock. Here we demonstrate that deletion of the DHS-1-P phosphatase YSR2 induced thermotolerance. Goffleb et al. [25] demonstrated that deletion of the DHS-1-P lyase also induced thermotolerance. Since DHS-1-P is the only common sphingolipid accumulated in the DHS-1-P phosphatase deletion and lyase deletion mutants, it may be critical for induction of thermotolerance. In addition, Dickson et al. [21] demonstrated that both DHS and heat induced expression of the genes containing the global stress response element STRE. TPS2, trehalose biosynthesis gene, is one of these genes. It is induced by DHS and is thought to protect cells from heat stress [21]. It remains to be investigated whether a defect in DHS-1-P phosphatase increases heat tolerance through activating expression of these genes.

Another interesting aspect of this study is that YSR2 and YSR3 appear to be functionally distinct. For example, overexpression of YSR2 inhibited cell proliferation more significantly than that of YSR3. Deletion of YSR2 but not YSR3 conferred thermotolerance. These differences in physiological functions resulted from the different roles that YSR2 and YSR3 have in regulation of metabolism of sphingolipids. One reason why YSR2 and YSR3 have different roles in controlling metabolism of sphingolipids may be due to their different expression levels. YSR3 has low mRNA levels, thus suggesting that it may have a less significant role in sphingolipid metabolism and stress responses. The differences may also be due to different protein stability, or different specificity to DHS or other substrates. This has yet to be investigated.

Mandala et al. [29] have demonstrated that deletion of *YSR2* conferred thermotolerance. Our results are in agreement with this. They also demonstrated that deletion of *YSR3/LBP2* significantly induced thermotolerance, which our results do not support. Interestingly, we found that when the *URA3* gene was used as a selectable marker to delete the *YSR3* gene, it conferred resistance to heat stress. Introduction of the *URA3* gene alone into the uracil auxotrophy mutant (JK9-3d α) also conferred thermotolerance (Table 3). When we deleted *YSR3* with the G418 resistance gene, the mutant did not show a thermotolerance phenotype. In the study by Mandala et al. [29], the *URA3* gene was used as the selectable marker to disrupt *YSR3/LBP2*, thus explaining the differences observed. Therefore, an increase in

thermotolerance of Δ*ysr3-1* is due to gain of the *URA3* gene function, not due to deletion of *YSR3*. Accordingly, the conclusion that deletion of *YSR3*/*LBP2* induces thermotolerance needs to be readdressed.

In summary, this study shows that YSR2 and YSR3 differentially mediate cell proliferation and heat-stress response by regulating sphingolipid metabolism in the yeast *S. cerevisiae*, indicating that a role for sphingolipids in mediating stress responses is evolutionarily conserved from the yeast *S. cerevisiae* to mammalian cells.

L.M.O. is the recipient of a Paul Beeson Physician Faculty Scholars in Aging Research Award. We thank Dr. Yusuf Hannun for helpful discussions and critique of this manuscript. We also thank Dr. Joseph Heitman for help with this manuscript, Dr. Alicja Bielawska for assistance in lipid analysis, and Ms Erlina Siragura for assistance in FACS analysis. This work was supported by Grant AG-12467-05 (L.M.O.).

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Received 22 February 1999/30 April 1999; accepted 9 June 1999