

# Cloning of an Alkaline Ceramidase from *Saccharomyces cerevisiae*

AN ENZYME WITH REVERSE (CoA-INDEPENDENT) CERAMIDE SYNTHASE ACTIVITY\*

(Received for publication, November 2, 1999, and in revised form, December 4, 1999)

Cungui Mao<sup>‡§</sup>, Ruijuan Xu<sup>‡§</sup>, Alicja Bielawska<sup>¶</sup>, and Lina M. Obeid<sup>‡¶||</sup>

From the <sup>‡</sup>Division of General Internal Medicine at the Ralph H. Johnson Veterans Administration Hospital and the Departments of Medicine and <sup>¶</sup>Biochemistry at the Medical University of South Carolina, Charleston, South Carolina 29425

**Ceramide is not only a core intermediate of sphingolipids but also an important modulator of many cellular events including apoptosis, cell cycle arrest, senescence, differentiation, and stress responses. Its turnover may be tightly regulated. However, little is known about the regulation of its metabolism because most enzymes responsible for its synthesis and breakdown have yet to be cloned. Here we report the cloning and characterization of the yeast gene *YPC1* (*YBR183w*) by screening *Saccharomyces cerevisiae* genes whose overexpression bestows resistance to fumonisins B1. We demonstrate that the yeast gene *YPC1* encodes an alkaline ceramidase activity responsible for the breakdown of dihydroceramide and phytoceramide but not unsaturated ceramide. *YPC1* ceramidase activity was confirmed by *in vitro* studies using an *Escherichia coli* expression system. Importantly, *YPC1p* also has reverse activity, catalyzing synthesis of phytoceramide from palmitic acid and phytosphingosine. This ceramide synthase activity is CoA-independent and is resistant to fumonisins B1, thus explaining why *YPC1* was cloned as a fumonisins B1-resistant gene.**

Ceramide forms the backbone of complex sphingolipids in mammalian cells. Ceramide, its breakdown product sphingosine, and its metabolite sphingosine-1-P are important signaling molecules that mediate different cellular events including apoptosis, growth arrest, stress responses, and proliferation (see reviews Refs. 1–4). As a signaling molecule, turnover of ceramide must be tightly regulated. Ceramide synthase and ceramidase catalyze synthesis and degradation of ceramide, respectively.

Ceramide synthase activity has been detected in mammalian (5) and yeast cells (6). It catalyzes the acylation of sphingosine by a fatty acyl-CoA. Additional studies show that ceramide can also be synthesized *in vitro* from sphingosine and palmitic acid in a CoA-independent reverse activity of a sphingolipid ceramide deacylase (7) or an alkaline ceramidase (8). To date no ceramide synthase has been purified or cloned.

Several ceramidase activities responsible for the breakdown

of ceramide have been detected in mammalian cells (9). Ceramidases are classified as acidic, neutral, or alkaline based on their pH optimum. Acidic ceramidase is localized in lysosomes and is responsible primarily for catabolism of ceramide (10). This enzyme has been identified, and its cDNA has been cloned from human (11) and mouse (12). On the other hand neutral and alkaline ceramidases have been implicated in signal transduction and cell regulation (13, 14). One of these enzymes has recently been characterized and purified to homogeneity (15).

As in mammals, dihydroceramide and phytoceramide are important intermediates of complex sphingolipids in the yeast *Saccharomyces cerevisiae* (4). Several studies have implicated dihydro/phytoceramide, dihydro/phytosphingosine, and dihydro/phytosphingosine 1-phosphate in mediating regulation of cell growth and stress responses in yeast (16–18). Consequently, cellular levels of these sphingolipids have to be regulated either by their immediate conversion to more complex sphingolipids or by their breakdown. Coordinated action of multiple metabolic enzymes is a key to fulfill this task of regulation and underscores the importance of this regulation for signaling processes in *S. cerevisiae*. Identification of these enzymes will facilitate our understanding of the signaling processes mediated by sphingolipids.

We elected to apply yeast genetic techniques to clone ceramidase synthase. This enzyme is also a target of a fungal toxin, namely fumonisins B1, which inhibits cell growth by suppressing synthesis of sphingolipids (6). We therefore used the strategy of overexpressing a high copy yeast genomic DNA library to identify genes whose overexpression endows resistance to growth suppression by fumonisins B1. By using this strategy we identified a gene whose overexpression imparts on cells the ability to synthesize sphingolipids in the presence of fumonisins B1. Here we describe this approach, and we provide evidence that the gene we obtained, *YPC1*,<sup>1</sup> in fact encodes a yeast alkaline ceramidase. Moreover, *YPC1p* also has the reverse activity of catalyzing ceramide formation from phytosphingosine and palmitic acid independent of coenzyme A, thus explaining why we obtained this enzyme as a fumonisins B1-resistant gene.

## MATERIALS AND METHODS

**Yeast and Bacterial Strains**—Yeast strains used in this study are listed in Table I. The mutant strain *Δyor1* was derived from the wild type JK9–3d  $\alpha$  by replacing a portion (from the start codon to nt 1073) of the *YOR1* coding sequence with the G418-resistant gene (KanMax) as

\* This work was supported in part by National Institutes of Health Grants AG16583 and AG12467. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF191745.

§ Both authors contributed equally to this work.

|| To whom correspondence should be addressed: Division of General Internal Medicine, 114 Doughty St., Rm. 602 STB, P. O. Box 250779, Charleston, SC 29425. Tel.: 843-953-3484; Fax: 843-953-0843; E-mail: obeidl@muscd.edu.

<sup>1</sup> The abbreviations used are: *YPC1*, the Yeast Phyto-ceramidase gene; *YPC1p*, the product of the *YPC1* gene; G418, geneticin; nt, nucleotide; PCR, DNA polymerase chain reaction; ORF, open reading frame; DHS, dihydrosphingosine; DHS-1-P, dihydrosphingosine 1-phosphate; PHS, phytosphingosine; PHS-1-P, phytosphingosine 1-phosphate; IPC, inositol phosphoceramide; MIPC, mannosylated IPC; ER, endoplasmic reticulum.

TABLE I  
Yeast strains used in this study

Strain	Genotype	Source
JK9-3d a	<i>MAT a leu2-3,112 ura3-52 rme1 trp1 his4 HMLa</i>	Mao et al. (18)
JK9-3d α	<i>MAT α leu2-3,112 ura3-52 rme1 trp1 his4 HML a</i>	Mao et al. (18)
Δ <i>yor1</i>	<i>MAT α leu2-3,112 ura3-52 rme1 trp1 his4 yor1Δ::KanMax4 HML a</i>	This study
Δ <i>ypc1</i>	<i>MAT α leu2-3,112 ura3-52 rme1 trp1 his4 ypc1Δ::KanMax4 HML a</i>	This study
Δ <i>yor1</i> -Vec	Derivative of Δ <i>yor1</i> transformed with the vector pYES2	This study
Δ <i>yor1</i> -Vec	The derivative of Δ <i>yor1</i> transformed with the vector pYES2	This study
Δ <i>yor1</i> -YPC1	The derivative of Δ <i>yor1</i> transformed with pYES2-YPC1	This study

described (19). Wild type and mutant strains were grown in YPD medium (1% yeast extract, 2% peptone, and 2% dextrose, Difco). Plasmid-containing strains were grown in a uracil dropout medium SC-ura (CLONTECH). SC-ura medium with fumonisin B1 (450 μM) and Nonidet P-40 (0.005%) purchased from Sigma were used for selection of fumonisin B1-resistant transformants. SC-ura medium with 2% galactose was used to induce gene expression under control of the *Gal1* promoter in the vector pYES2. *Epicurian coli* XL-1 and SURE strains were used for most gene manipulations. *E. coli* Top10 strain was used for expression of yeast proteins.

**Disruption of the YPC1 Gene**—The *YPC1* gene was replaced by a disruption construct consisting of the G418-resistant gene (KanMax) flanked by 5' end (nt 16–46) and 3' end (nt 892–926) of the *YPC1* coding region as described (19). The diploid cells containing the *ypc1* deletion allele were sporulated, and the resulting tetrads were dissected as described (18). The haploid strain (Δ*ypc1*) harboring the *ypc1* deletion allele was selected by resistance to G418, and deletion of *YPC1* was verified by PCR using a primer upstream of the start codon of the *YPC1* coding region and another primer located in the middle of the gene KanMax.

**Construction of the Yeast Genomic DNA Library**—DNA was isolated from the yeast strain Δ*yor1* as described (20). To remove RNA, RNase (free from DNase I) was added to DNA preparations and incubated at 37 °C for 30 min. DNA was partially digested with the diluted *Sau31A* for 20 min. *Sau31A*-digested DNA was run on 0.8% agarose gel, and 4–6-kilobase DNA fragments were excised and purified by a Ultra-Free-DA unit (Amicon) as suggested by the manufacturer. After being extracted with phenol:chloroform:isoamyl alcohol (25:24:1) followed by chloroform using a phase lock gel tube (5' → 3', Inc., Boulder, CO), DNA was concentrated and desalted by a Microcon 100 device (Amicon) and quantitated by an UV spectrometer (Beckman) using a built-in DNA analysis program. The DNA concentration was adjusted to 0.5 μg/μl with 0.5× TE buffer (5 mM Tris-HCl, pH 7.4, containing 0.5 mM EDTA). The purified DNA (50 ng) was ligated at 14 °C overnight to 50 ng of the plasmid YEplac195 (21) which was digested by *Bam*HI and dephosphorylated by the shrimp alkaline phosphatase (Roche Molecular Biochemicals). The ligation reaction (10 μl) was desalted by the Microcon 100 device, and 1/5 of the ligated DNA was transformed into SURE2-competent cells (Stratagene) by electroporation at 17 kV/cm field strength, 200 megohms resistance, 25 microfarads capacitance. Transformants were selected on LB plates with 75 μg/ml ampicillin. A total of 5 × 10<sup>5</sup> transformants was obtained, with 98% of them having inserts based on a DNA digestion test. All transformants were pooled and spun down at 4,000 rpm in a 150-ml centrifuge bottle for 15 min at 4 °C. Plasmids were purified using a Maxi-prep kit (Qiagen) after cells were washed twice with ice-cold TE buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA).

**Cloning of the YPC1 Gene**—The *S. cerevisiae* strain Δ*yor1* was transformed with the above genomic DNA library by a modified lithium acetate method (22), and transformants were selected for uracil prototrophs. Approximately a total of 1 × 10<sup>6</sup> transformants was obtained and pooled. After cells were diluted to a density of 1 × 10<sup>6</sup> cells/ml, 0.2 ml of cells was plated onto each of 5 SC-ura plates containing 450 μM fumonisin B1 and 0.005% Nonidet P-40. The plates were incubated at 30 °C for 4 days. More than 100 transformants that survived on fumonisin B1 plates were obtained. Twenty transformants were chosen for further analysis. Plasmids were isolated from these 20 transformants as described (20) and transformed into the *E. coli* SURE strain by electroporation. Plasmids were amplified and purified as described (20).

To confirm that resistance to fumonisin B1 was due to overexpression of a gene in a plasmid, each plasmid was re-introduced into the Δ*yor1* strain. The transformants were tested for resistance to fumonisin B1 by growing on the fumonisin B1 plates. Inserts in the plasmids that gave resistance to fumonisin B1 were partially sequenced from both ends using primers 5'CAGCTATGACCATGATTACGCC3' (forward) and 5'ACGTTGTAAAACG ACGGCCAGTG3' (reverse). Searching the *Saccharomyces* Genomic Data base (SGD) with the partial sequences derived the entire sequences of inserts.

**Plasmid Construction for Protein Expression in Yeast**—The open reading frame (ORF) of the *YPC1* gene was amplified by PCR (1 cycle of 94 °C for 2 min; 30 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min; 1 cycle of 72 °C for 10 min) using the precision plus *Taq* polymerase (Stratagene, Inc.) and the primers 5'CGGGGTACCATGG-GAATATTCGTTGGAACATATC3' (forward) and 5'CGGGAATTCTTACTTCTCCTTTTAACTTCAATTGATTGATC3' (reverse). The amplified products were digested by restriction enzymes *Kpn*I and *Eco*RI and cloned into *Kpn*I and *Eco*RI sites of the vector pYES2 to yield the construct pYES2-YPC1, thus expressing YPC1p under control of the promoter *Gal1*. The construct pYES2-YPC1 and the empty vector pYES2 were introduced into the strain Δ*yor1* by the lithium acetate method after sequencing to ensure that no errors were introduced into the *YPC1* ORF by PCR. The strain containing pYES2-YPC1 or pYES2 was grown and maintained in SC-ura medium with 2% glucose. Expression of YPC1p was induced in SC-ura medium with 2% galactose.

**Expression of YPC1p in E. coli**—The coding sequence of the gene *YPC1* was excised by restriction enzymes *Kpn*I and *Eco*RI from the plasmid pYES2-YPC1 and cloned into *Kpn*I and *Eco*RI sites of the vector pBAD/His B (Invitrogen) which was digested by the same enzymes to create pBAD/His-YPC1, thus expressing the polyhistidine (His) and Xpress tagged YPC1p under control of the *ara*BAD promoter (P<sub>BAD</sub>). pBAD/His and pBAD/His-YPC1 were introduced into the *E. coli* strain TOP10 by electroporation. Expression of the tagged YPC1p was induced by 0.2% arabinose whose concentration was optimized as suggested by the manufacturer. Expression of the tagged YPC1p was verified by Western blotting analysis using the mouse monoclonal antibody against the Xpress or His tag (Invitrogen, Inc.). The tagged YPC1p was extracted from *E. coli* cells by treating with lysozyme in a lysis buffer (25 mM Tris-HCl, 0.1 mM phenylmethylsulfonyl fluoride and 20 μg/ml CLAP (CLAP, the mixture of chymostatin, leupeptin, aprotinin, and pepstatin)) followed by several cycles of freezing and thawing and a brief sonication according to the procedures suggested by the manufacturer. The tagged YPC1p was purified by a Talon metal affinity column (CLONTECH, Inc.) according to procedures provided by the manufacturer.

**Preparation of Radiolabeled Ceramides**—[<sup>3</sup>H]Ceramide and phytoce-ramide were synthesized as follows: *N*-[9,10-<sup>3</sup>H]D-erythro-C16-ceramide and *N*-[9,10-<sup>3</sup>H]D-ribo-C16-phytoceramide were prepared by acylation of the respective sphingoid bases with [9,10-<sup>3</sup>H]palmitoylchloride generated *in situ* from [9,10-<sup>3</sup>H]palmitic acid as described (23). D-Erythro sphingosine was obtained in stereo- and enantio-specific synthesis as described previously (24). Phytosphingosine was from Sigma. *N*-Hexanoyl-D-erythro-[4,5-<sup>3</sup>H]dihydrosphingosine ([<sup>3</sup>H]-C-6-dihydroce-ramide), D-erythro-[4,5-<sup>3</sup>H]dihydrosphingosine, and D-erythro-[4,5-<sup>3</sup>H]dihydrosphingosine 1-phosphate were from American Radiolabeled Chemicals (ARC, Inc.).

**Preparation of Microsomes**—Microsomes were isolated from the yeast cells as described (20) with minor modification as follows. Cells were suspended in a lysis buffer (25 mM Tris-HCl, pH 7.4, containing 0.1 M phenylmethylsulfonyl fluoride and 20 μg/ml CLAP) after being washed twice with ice-cold water. Cells were homogenized three times (3 × 30 s) with acid-washed glass beads (Sigma) using a Mini-bead-beater-8 (Biospec Products) set at the maximum speed. The cells were chilled on ice for 1 min between homogenizations. The resulting supernatant was transferred to a new tube after centrifugation at 2,000 rpm for 5 min. Unbroken cells and cell debris were removed by centrifugation at 4,000 rpm for 5 min. To pellet the membrane fraction, the supernatant was centrifuged at 40,000 rpm for 40 min at 4 °C. The membrane fraction was rinsed gently with the lysis buffer and suspended in an appropriate assay buffer. Protein concentrations were determined using a Bradford reagent (Bio-Rad).

**Measurements of Ceramidase Activity and Its Reverse Activity**—[<sup>3</sup>H]Ceramide (2.75 nmol), [<sup>3</sup>H]phytoce-ramide (2.5 nmol), or [<sup>3</sup>H]C-6-dihydroce-ramide (0.2 nmol) in ethanol was dried on a SpeedVac (Savant) and dissolved in 20 μl of the assay buffer A (25 mM Tris-HCl, pH 8.0, 5 mM CaCl) with 0.5% Triton X-100 by boiling for 10 s followed by sonication in a water bath ultrasonicator (Cole-Parmer) for 2 min as described (25). To measure ceramidase activity, 20 μl (approximately

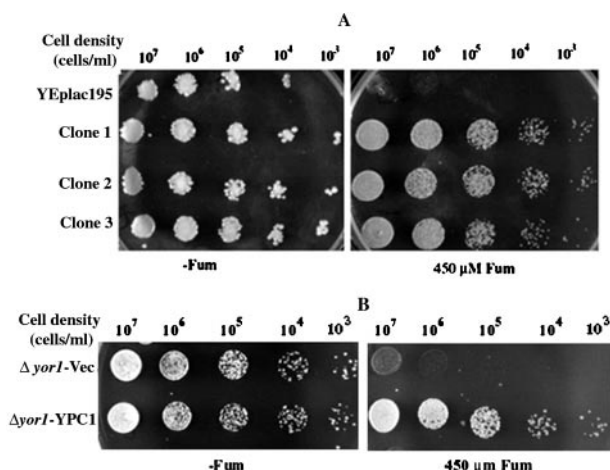


FIG. 1. Overexpression of YPC1 bestows resistance to fumonisin B1. Yeast cells grown to the late log phase in SC-ura medium were serially diluted. Three  $\mu$ l of cells from each dilution were spotted onto SC-ura medium with or without fumonisin B1 and incubated at 30 °C for 3 days. The plates were photographed on a ChemImage system (Alpha-Inotech). A, cells with an empty vector (YEplac195) or with the vector containing inserts (clones 1–3) were grown on SC-ura plates containing 2% glucose; B, cells of the strain  $\Delta$ yor1-Vec or  $\Delta$ yor1-YPC1 were grown on SC-ura plates containing 2% galactose. Fum, fumonisin B1.

150–300  $\mu$ g of proteins) of microsomes were added to substrates, and reactions were incubated at 30 °C for 40–90 min. Both the protein concentration and the time of incubation were within the linear range for the assay. The reactions were terminated by adding 200  $\mu$ l of chloroform:methanol (2:1, v/v). Reaction mixture was dried and dissolved in 30  $\mu$ l of chloroform:methanol (2:1, v/v). 25  $\mu$ l of the mixture was applied onto a silica gel 60A TLC plate (Whatman) and resolved by the solvent system I (chloroform, methanol, 25% ammonium hydroxide, 9:2:0.5, v/v) (25). Free palmitic acid or DHS, the reaction product separated from the substrate by TLC, was identified according to a standard and quantified by a scintillation counter. One unit of ceramidase was defined as the amount of the enzyme needed to release 1 pmol of palmitic acid or dihydro sphingosine per min.

To measure the activity of ceramide synthase independent of fatty acyl-CoA, phytosphingosine (5 nmol) and [ $^3$ H]palmitic acid (0.3 nmol) were dried on a SpeedVac and dissolved in 20  $\mu$ l of the buffer A as described above. Twenty  $\mu$ l of microsomes were added to the above substrates and incubated at 30 °C for 4–5 h. Both protein concentration and time of incubation were within the linear range for the assay. Reactions were terminated as described above. Ceramide formation was analyzed by the same TLC system with C16-phytoceramide as a standard. One unit of ceramide synthase was defined as the amount of the enzyme needed to form 1 pmol of phytoceramide per min.

**Sphingolipid Labeling**—Cells ( $3 \times 10^7$  in 1 ml of medium) were labeled with [ $^3$ H]palmitic acid, serine, or C-6-dihydroceramide (5–10  $\mu$ Ci) at 30 °C for different periods. Total lipids were extracted, deacylated by monomethylamine (20% in ethanol), and resolved by TLC using the solvent system II (chloroform, methanol, 4.2 N ammonium hydroxide, 9:7:2, v/v) as described (26). TLC plates were sprayed with EN $^3$ HANCE and radiographed on BioMax films (Eastman Kodak Co.). Radiolabeled sphingolipids were identified according to the authentic standards included on the same TLC plate. To quantify an individual lipid, the radioactive bands were scraped and counted by a scintillation counter (Beckman Instruments).

**Protein Analysis**—Proteins were separated by SDS-polyacrylamide gel electrophoresis and were detected by Western blotting analysis by following standard procedures.

## RESULTS

**Overexpression of YPC1 Imparts Resistance to Fumonisin B1**—In order to clone the *S. cerevisiae* ceramidase, we set out to screen for genes whose overexpression imparts resistance to fumonisin B1, using a high copy yeast genomic DNA library. The yeast strain we used for this screen had a deletion of the gene that encodes the long chain base lyase, which made this strain more sensitive to fumonisin B1 and allowed us to

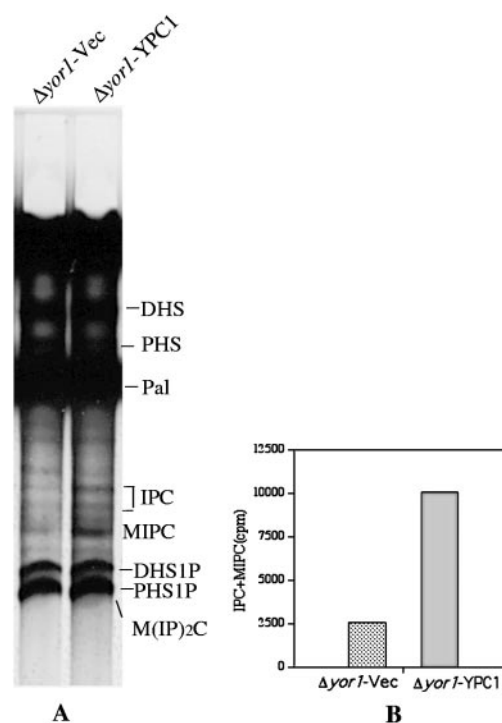


FIG. 2. Overexpression of YPC1 rescues from fumonisin B1 inhibition. The  $\Delta$ yor1-Vec or  $\Delta$ yor1-YPC1 cells ( $3 \times 10^7$ ) grown in SC-ura medium with 2% galactose which induced expression of YPC1 were labeled with 10  $\mu$ Ci of [ $^3$ H]palmitic acid (Pal) (43 Ci/mmol) in the presence of 100  $\mu$ M fumonisin B1 as described under “Materials and Methods.” Total lipids were extracted and resolved by TLC after base hydrolysis by monomethylamine. A, a representative radiograph of the TLC of several experiments on a BioMax x-ray film; B, quantitation of complex sphingolipids (IPC and MIPC).

use less fumonisin B1 for the screen. Upon screening this library we obtained 3 clones all of which were similar and encoding for a known yeast gene that is a member of the ATP-binding cassette (ABC) transporter family of genes namely *YOR1* (yeast oligomycin resistance gene). The reason *YOR1p* endows resistance to fumonisin B1 appears to be due to its ability to pump out fumonisin B1 and/or other sphingolipids that may accumulate as a result of fumonisin B1 treatment.<sup>2</sup>

In order to pursue our initial goal to obtain ceramide synthase, we next elected to prepare a yeast genomic DNA library from the  $\Delta$ yor1 strain and to use this library to screen for genes that impart resistance to fumonisin B1 that are different from *YOR1* as described under “Materials and Methods.” Of the 20 fumonisin B1-resistant transformants we analyzed, 16 were found to have plasmid-dependent resistance to fumonisin. Fig. 1A shows that cells transformed with three separate representative clones confer resistance to fumonisin B1. The inserts of the plasmids were sequenced from both ends, and a search of the *Saccharomyces* Genomic Data base with the partial sequences obtained derived the entire sequence of the inserts. Sequencing revealed that 7 out of 16 clones were identical, having the same insert, spanning three ORFs. The 9 other plasmids contained different inserts. However, all the clones shared a consensus sequence covering a hypothetical ORF *YBR183w* on chromosome II. *YBR183w*, designated as *YPC1*, was the only ORF in one of the plasmids. We speculated that *YBR183w* might confer resistance to fumonisin B1. To verify if that was the case, we amplified the ORF *YBR183w* from yeast genomic DNA by PCR and cloned it into the yeast expression vector pYES2 to create the plasmid pYES2-YPC1, thus ex-

<sup>2</sup> C. Mao and L. M. Obeid, manuscript in preparation.

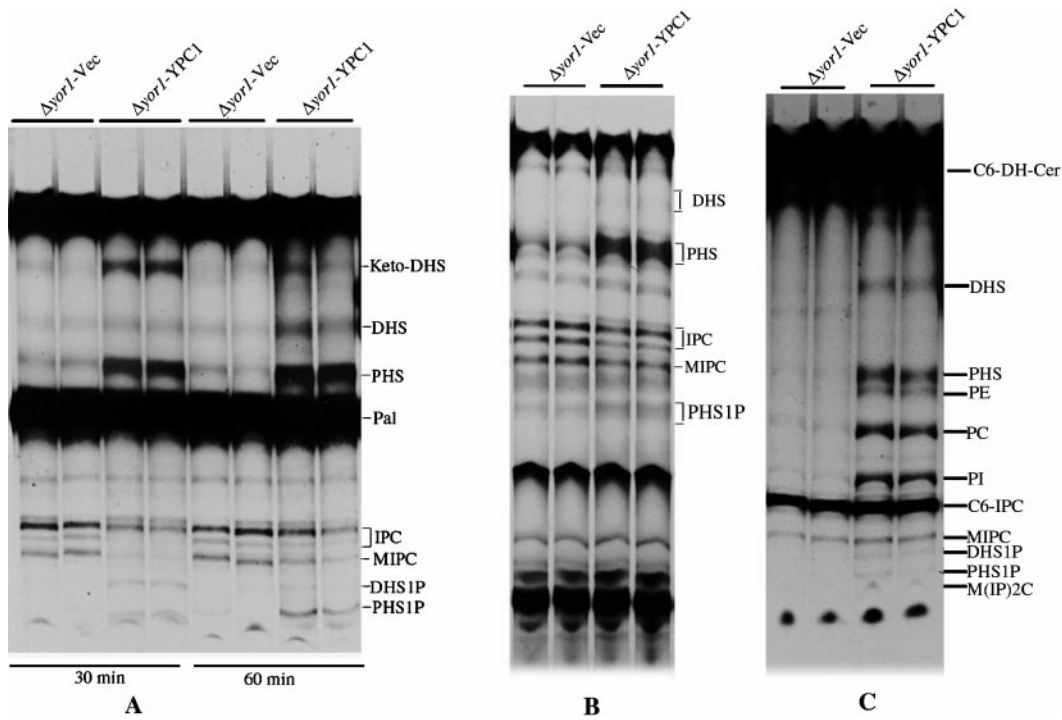


FIG. 3. **Overexpression of YPC1 causes an increase in breakdown of sphingolipids.** Cells ( $3 \times 10^7$ ) of the strain  $\Delta$ yor1-Vec and  $\Delta$ yor1-YPC1 were labeled with 10  $\mu$ Ci of palmitic acid (Pal) (A), 5  $\mu$ Ci of serine (B), or 2.5  $\mu$ Ci of C-6-dihydroceramide (C) as described under "Materials and Methods." Base hydrolysis of lipid extracts with monomethylamine was performed in A and B but not in C. Lipids were resolved by TLC. Dihydrosphingosine (DHS) and dihydrosphingosine-1-P (DHS1P) were identified by radiolabeled standards, whereas other sphingolipids or phospholipids were identified by non-radiolabeled standards.

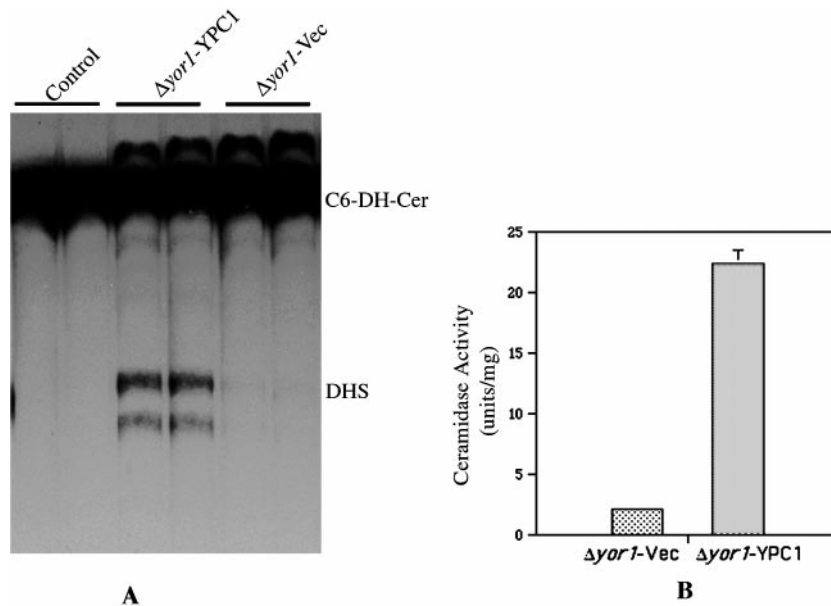


FIG. 4. **Overexpression of YPC1 causes an increase in ceramidase activity.** Microsomes were prepared as described under "Materials and Methods" from the  $\Delta$ yor1-Vec strain and the  $\Delta$ yor1-YPC1 strain in which YPC1p was overexpressed under the control of the *Gal1* promoter. To measure ceramidase activity, microsomes from the strains were added to 200 pmol of *N*-hexanoyl-D-erythro-[4,5- $^3$ H]dihydrosphingosine ( $^3$ H]-C-6-dihydroceramide) as described under "Materials and Methods." Reactions were performed at 30  $^{\circ}$ C for 90 min as described under "Materials and Methods." The heat-inactivated microsomes of the vector containing cells was used as a blank control. A, reaction products were resolved by TLC and autoradiographed. Dihydrosphingosine was quantitated by scraping and counting. B, ceramidase activity was determined by subtracting background counts from the blank control. The data represent the mean of an experiment performed in duplicate and are representative of at least three independent experiments. C6-DH-Cer, C-6-dihydroceramide; DHS, dihydrosphingosine.

pressing YPC1p under the control of the *Gal1* promoter. pYES2-YPC1 and the vector pYES2 were introduced into the strain  $\Delta$ yor1, respectively. The vector-containing strain ( $\Delta$ yor1-Vec) and the pYES2-YPC1-containing strain ( $\Delta$ yor1-YPC1) were grown in SC-ura medium with 2% galactose. The expression of the gene YPC1 was induced by galactose. Fig. 1B shows

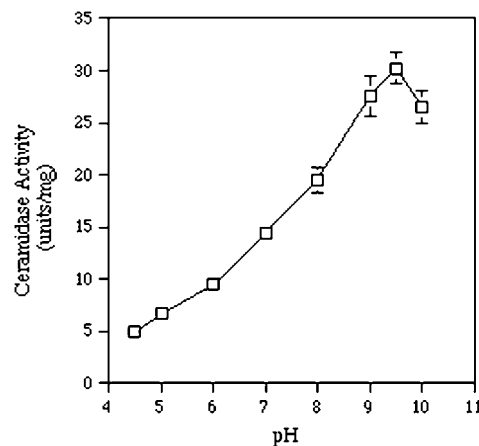
that the strain  $\Delta$ yor1-YPC1 but not  $\Delta$ yor1-Vec was resistant to fumonisins B1 in the galactose-containing medium. These data suggest that the resistance to fumonisins B1 is indeed attributed to overexpression of YPC1p.

*Overexpression of YPC1p Changes the Metabolism of Sphingolipids*—Examination of the YPC1p sequence predicted it to

have several transmembrane domains. It had high homology to another yeast hypothetical protein encoded by the ORF *YPL087w*. A BLAST search showed that YPC1p did not have any significant homology to other proteins with known functions in all protein data bases searched, suggesting it is a novel protein. Motif search indicated that it contained an ATP-binding site (GXGXXG(X...X)<sub>10</sub>K, where X indicates any amino acid residue) shared by human diacylglycerol kinase  $\zeta$  (27) and many protein kinases. On its carboxyl terminus, it had an ER retention signal (KKXK), which is shared by several ER proteins including sphingolipid metabolic enzymes ketodihydrosphingosine reductase TSC10p (28) and dihydrosphingosine (or dihydroceramide) hydroxylase SYR2p (29), suggesting it might be localized to the ER.

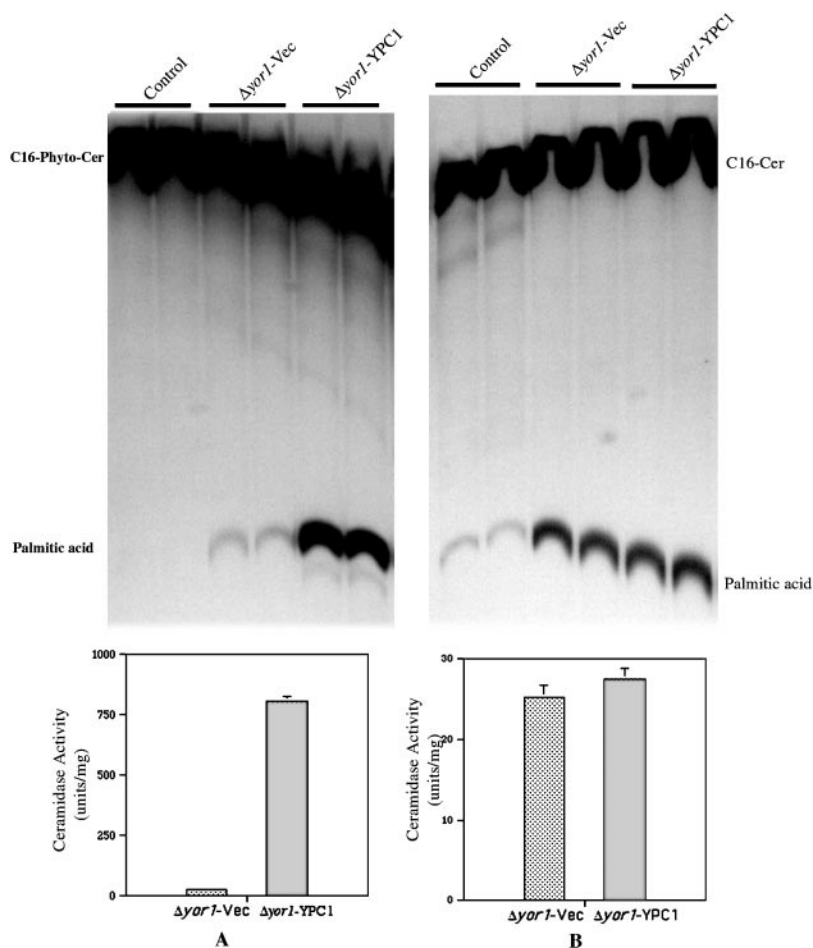
We could not predict function of the product of the *YPC1* gene based on protein homology. However *YPC1* was selected for allowing resistance to fumonisin B1. Fumonisin B1 inhibited cell growth in the DHS-1-P lyase (DPL1) deletion because it led to accumulation of phosphorylated long chain bases and/or because it inhibited formation of ceramide and sphingolipids.<sup>2</sup> Therefore, we speculated that overexpression of YPC1p could either lead to breakdown of accumulated long chain base phosphates or to bypassing the ceramide synthase block and generating ceramide and other ceramide-containing sphingolipids despite the presence of fumonisin B1. To investigate this, the strains  $\Delta yor1$ -Vec and  $\Delta yor1$ -YPC1 were grown in SC-ura medium with galactose, which induced YPC1p expression. The cells were then treated with 100  $\mu$ M fumonisin B1 for 2 h and labeled with palmitic acid for 4 h. Total lipids were extracted and resolved by TLC after base hydrolysis by monomethylamine as described under "Materials and Methods." Autoradiography of the TLC plate (Fig. 2A) demonstrated that forma-

tion of the sphingolipids IPC, MIPC, and M(IP)2C was significantly blocked by fumonisin B1 in the vector control cells ( $\Delta yor1$ -Vec) but not in the YPC1p-overexpressing cells ( $\Delta yor1$ -YPC1). Quantitation showed that the  $\Delta yor1$ -YPC1 cells had 4 times more incorporation of palmitic acid into complex sphingolipids (IPC, MIPC, and M(IP)2C) than did the  $\Delta\Delta yor1$ -Vec cells in the presence of fumonisin B1 (Fig. 2B). The data sug-



**FIG. 5. YPC1p has alkaline ceramidase activity.** The activity that hydrolyzed [<sup>3</sup>H]C-6-dihydroceramide was measured as described in Fig. 4 with microsomes isolated from cells overexpressing YPC1p at different pH values. Sodium acetate buffer (25 mM) was used for pH 4.5–6, Tris-HCl buffer (25 mM) was used for pH 7–8, and glycine NaOH buffer (25 mM) was used for pH 9–10. All buffers contained 5 mM CaCl<sub>2</sub> and 0.25% Triton X-100. The data represent the mean of an experiment performed in duplicate and are representative of two independent experiments.

**FIG. 6. YPC1 hydrolyzes phytoceramide but not unsaturated ceramide.** Microsomes were assayed for ceramidase activity from the  $\Delta yor1$ -Vec and  $\Delta yor1$ -YPC1 cells toward phytoceramide (A) or ceramide (B) as described in Fig. 4. Reactions were performed at 30 °C for 40 min. *Upper panels*, the autoradiograph of the TLC; *bottom panels*, ceramidase activity. The data represent the mean of an experiment performed in duplicate and are representative of two independent experiments. *C16-Phyto-Cer*, C16-phytoceramide; *C16-Cer*, C16-ceramide.



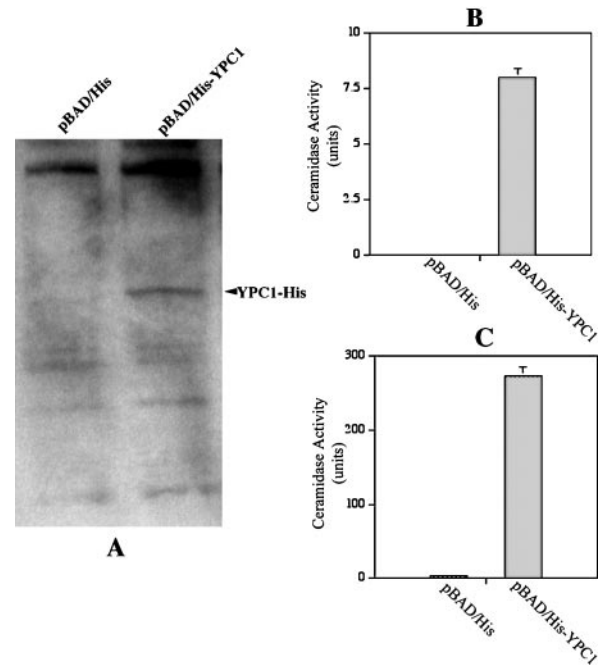
gest that overexpression of YPC1p reverses fumonisin B1-induced growth inhibition by allowing synthesis of sphingolipids.

To gain further insight into the function of YPC1p, we next examined the effect of overexpression of YPC1p on sphingolipid metabolism in the absence of fumonisin B1. Upon labeling with either palmitic acid (Fig. 3A) or serine (Fig. 3B), we saw the opposite effect from above. The strain  $\Delta yor1$ -YPC1 had a reduced rate of radiolabel incorporation into complex sphingolipids as compared with the strain  $\Delta yor1$ -Vec. On the other hand the  $\Delta yor1$ -YPC1 strain accumulated PHS, DHS, PHS-1-P, and DHS-1-P much more than the  $\Delta yor1$ -Vec (Fig. 3, A and B). These data raised an intriguing possibility that YPC1p has a dual function of enhancing synthesis or breakdown of sphingolipids, depending on the presence or absence of fumonisin B1.

**YPC1 Enhances Cellular Ceramidase Activity**—Next we set out to investigate the dual activity of YPC1p. Decreases in IPC and other complex sphingolipids and increases in DHS, PHS, and their phosphorylated products upon overexpression of YPC1p hinted that YPC1p might be mediating breakdown of either ceramides or more complex sphingolipids. We therefore investigated the ability of YPC1 to mediate breakdown of ceramides by labeling YPC1p-overexpressing cells with C-6-dihydroceramide labeled with  $^3\text{H}$  at both the C-4 and C-5 positions of dihydrosphingosine backbone. We used C-6-dihydroceramide because it is taken up better than long acyl chain ceramides. Fig. 3C demonstrates that overexpression of YPC1p significantly enhanced degradation of dihydroceramide into DHS, PHS, DHS-1-P, and PHS-1-P. There were concomitant increases in phosphatidylinositol, phosphatidylcholine, and phosphatidylethanolamine. DHS is the immediate breakdown product of dihydroceramide, whereas PHS may be the product of DHS hydroxylation or the breakdown product of phytoceramide, which in turn is converted from dihydroceramide. Both DHS and PHS are known to be quickly phosphorylated by long chain base kinases to yield DHS-1-P and PHS-1-P, respectively (20). These are then quickly broken down by the lyase DPL1, and their products are incorporated into glycerolipids (20). These data suggest that YPC1p indeed enhances cellular ceramidase activity. Taken together with the results obtained by labeling in the absence of fumonisin, these data suggest that enhancement of cellular ceramidase activity may be a main function of YPC1.

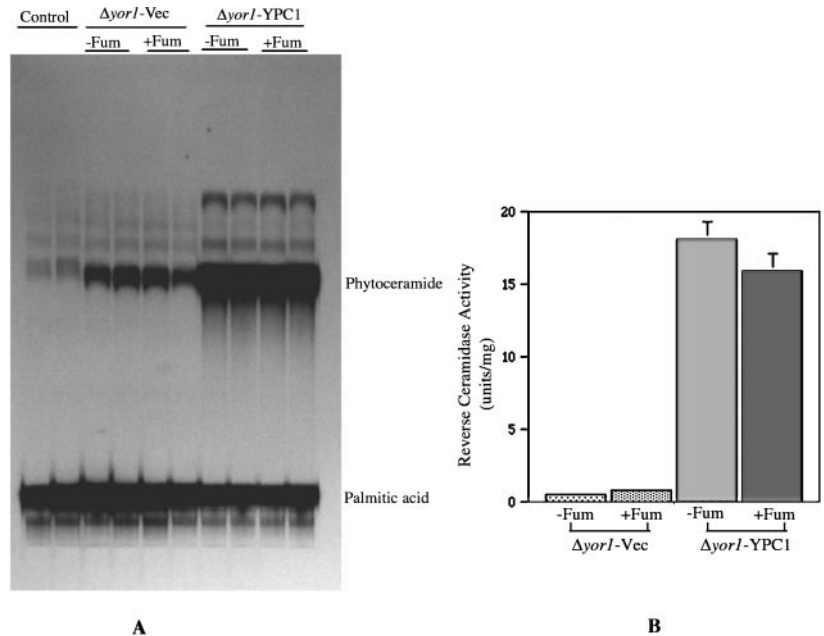
**YPC1 Has Ceramidase Activity**—We next set out to evaluate

if the increase in breakdown of cellular dihydroceramide upon overexpression of YPC1p was due to its ceramidase activity. Microsomes were prepared from the YPC1p-overexpressing cells ( $\Delta yor1$ -YPC1) or from the empty vector-containing cells ( $\Delta yor1$ -Vec). Microsomal preparations were then incubated with substrate, and the reactions were carried out at 30 °C for 90 min at pH 8.0. TLC was used to evaluate and quantitate product formation (Fig. 4A). When [ $^3\text{H}$ ]C-6-dihydroceramide was used as a substrate, microsomes from the  $\Delta yor1$ -YPC1 cells



**FIG. 7. YPC1 expressed in *E. coli* has ceramidase activity.** The His- and Xpress-tagged YPC1p expressed in *E. coli* Top10 cells was detected by Western blotting analysis using anti-Xpress antibody (A). Total soluble proteins were prepared from cells containing vector pBAD/His or pBAD/His-YPC1 and were applied to Talon metal affinity columns as described under “Materials and Methods.” Proteins bound to the columns were eluted, and 20- $\mu\text{l}$  eluants were assayed for ceramidase activity toward dihydroceramide (B) or phytoceramide (C) as described under “Materials and Methods.” The data represent the mean of an experiment performed in duplicate and are representative of two independent experiments.

**FIG. 8. YPC1 has ceramide synthase activity.** Microsomes from vector-containing cells ( $\Delta yor1$ -Vec) or YPC1-overexpressing cells ( $\Delta yor1$ -YPC1) were added to phytosphingosine (5.0 nmol) and [ $^3\text{H}$ ]palmitic acid (10  $\mu\text{Ci}$ , 0.3 nmol) and incubated at 30 °C for 5 h. The products of the reaction were resolved by TLC and autoradiographed (A). Phytoceramide was identified according to a standard as described under “Materials and Methods” and was quantitated by a scintillation counter. Total activity was determined by subtracting background counts of the blank control (B). -Fum is the activity in the absence of fumonisin B1; +Fum is the activity in the presence of 50  $\mu\text{M}$  fumonisin B1. The data represent the mean of an experiment performed in duplicate and are representative of four independent experiments.



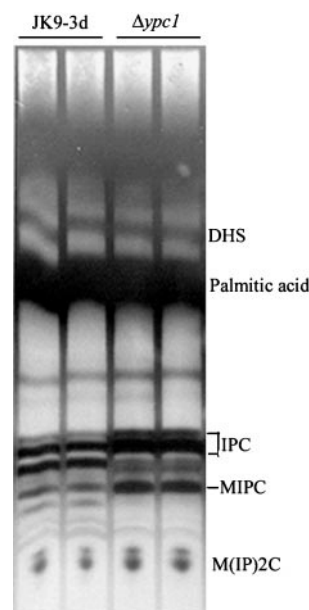
had 10 times more ceramidase activity than did microsomes prepared from the  $\Delta ypc1$ -Vec cells (Fig. 4B), thus indicating that YPC1p had ceramidase activity.

**YPC1 Has the pH Profile of an Alkaline Ceramidase Activity**—In order to determine to which ceramidase category this enzyme belongs, we next determined its pH profile. We performed ceramidase activity from microsomal preparations of the YPC1p-overexpressing cells in different buffers ranging in pH from 4.5 to 10. Fig. 5 shows that at pH 5.0 or lower, YPC1 had hardly any ceramidase activity. The activity increased with increase in pH peaking at a pH of 9.5. These data suggest that YPC1 encodes an alkaline ceramidase activity.

**YPC1 Increases Dihydro- and Phytoceramidase Activities but Not Unsaturated Ceramidase Activity**—Yeast cells have only dihydro- and phytoceramide but no ceramide. As demonstrated in Fig. 4, YPC1p had dihydroceramidase activity. We next determined the substrate specificity of YPC1p ceramidase activity toward phytoceramide and unsaturated ceramide. We tested microsomal preparations from cells overexpressing YPC1p compared with microsomal preparations from vector cells using  $^3\text{H}$ -labeled  $\text{C}_{16}$ -phytoceramide and  $\text{C}_{16}$ -ceramide. Microsomes from the YPC1-overexpressing cells had significantly increased activity toward phytoceramide as compared with vector cells (Fig. 6A). Interestingly microsomes from the YPC1-overexpressing cells demonstrated no increase in ceramidase activity over the vector-containing cells when unsaturated ceramide was used as substrate (Fig. 6B).

**Ceramidase Activity of YPC1 Expressed in E. coli**—To confirm that YPC1p indeed was a ceramidase and not a regulator of cellular ceramidase activity, we proceeded to heterologously express YPC1p in *E. coli*. To facilitate detection and purification of YPC1p, we tagged it with the epitope tag polyhistidine and X-press in an expression vector pBAD/His B (Invitrogen). The expression of the tagged YPC1p was induced in LB medium with 0.2% arabinose for 4 h. The tagged YPC1p was detected by Western blotting (Fig. 7A) using the antibody against X-press tag (Invitrogen). The His-tagged YPC1p was purified by a His-tagged protein purification kit. To test if the purified YPC1p has ceramidase activity an *in vitro* assay of the activity was carried out with dihydroceramide and phytoceramide as substrates. Fig. 7, B and C, demonstrates that YPC1p expressed in *E. coli* had high ceramidase activity at pH 8.0 using either substrate, suggesting that YPC1p is indeed an alkaline ceramidase.

**YPC1p Is a Dual Activity Ceramidase and Palmitoyl-CoA-independent Ceramide Synthase**—Despite the discovery that YPC1p is a *S. cerevisiae* alkaline ceramidase, we still wanted to reconcile this with the fact that overexpression of YPC1p endowed fumonisin B1 resistance. Evidence that YPC1p overexpression allowed yeast cells to synthesize sphingolipids in the presence of fumonisin B1 prompted us to determine whether YPC1p also has ceramide synthase activity. To test this we again used microsomes from cells overexpressing YPC1p or containing the empty vector pYES2 and measured *in vitro* ceramide synthase activity. When we utilized DHS or PHS and palmitoyl-CoA as substrates with microsomes isolated from YPC1p-overexpressing cells, no increase in ceramide synthase activity was detected as compared with microsomes from vector cells (data not shown). This implied that YPC1p was not a CoA-dependent ceramide synthase. Other reports (8, 25) have indicated that ceramide can be synthesized from sphingosine and palmitic acid in a CoA-independent manner and may actually be a result of the “reverse” reaction of ceramidase. Formation of ceramide from palmitic acid, and not from palmitoyl-CoA, was shown to be catalyzed by a hypothetical reverse activity of a ceramidase from rat brain (30), human kidney and



**FIG. 9. Deletion of YPC1 enhances synthesis of complex sphingolipids.** The same numbers ( $3 \times 10^7$ ) of cells of the mutant strain  $\Delta ypc1$  and the parental strain JK9-3d  $\alpha$  were labeled with [ $^3\text{H}$ ]palmitic acid for 2 h as described in Fig. 2. Total lipids were extracted and resolved by TLC after hydrolysis by monomethylamine. The labeled sphingolipids are indicated.

cerebellum (31), guinea pig epidermis (32), and most recently by a bacterial ceramidase (8) or a sphingolipid ceramide deacylase (25). We next examined whether YPC1p encoded such an activity by measuring ceramide formation using the same microsomal preparations as above with PHS and [ $^3\text{H}$ ]palmitic acid as substrates. Fig. 8, A and B, demonstrates that microsomes prepared from cells overexpressing YPC1p had 30-fold higher ceramide synthase activity; moreover, this increase in ceramide synthesis was not inhibited by the addition of fumonisin B1 to the *in vitro* reaction. These data suggest that YPC1p has an activity that catalyzes formation of ceramide from phytosphingosine and palmitic acid. This activity is distinct from the fatty acyl-CoA-dependent ceramide synthase activity that is inhibited by fumonisin B1. These data indicate that YPC1p is indeed a ceramidase with a reverse hydrolysis activity and prove the existence of such an enzyme.

**Deletion of YPC1 Reduces Complex Sphingolipids**—Finally, we wanted to evaluate phenotypes of the *ypc1* deletion mutant. We constructed a diploid strain with the *ypc1* null allele as described under “Materials and Methods.” Sporulation and tetrad dissection showed that deletion of YPC1 is viable, suggesting that the YPC1 gene is not an essential gene. In order to evaluate the endogenous function of YPC1p on metabolism of sphingolipids, we labeled the deletion mutant  $\Delta ypc1$  and its parental strain JK9-3d  $\alpha$  with [ $^3\text{H}$ ]palmitic acid. TLC analysis demonstrated that all complex sphingolipids labeled by [ $^3\text{H}$ ]palmitic acid increased in the  $\Delta ypc1$  strain compared with the wild type strain (Fig. 9). These data suggest that there is a block in the breakdown of these sphingolipids, presumably at the level of ceramidase. Ceramidase activity (hydrolyzing phytoceramide) from microsomal preparations of the  $\Delta ypc1$  strain was  $1.4 \pm 0.2$  units/mg as compared with  $22.0 \pm 1.7$  units/mg from the wild type strain JK9-3d  $\alpha$ . The CoA-independent (phyto-) ceramidase activity from the  $\Delta ypc1$  strain was not detected as compared with  $1.9 \pm 0.3$  units/mg from the wild type strain. These data demonstrate that deletion of YPC1 significantly reduced (phyto-) ceramidase activity and obliterated CoA-independent (phyto-) ceramidase activity.

Taken together, these results suggest that YPC1p indeed functions as an endogenous ceramidase in cells.

#### DISCUSSION

In this study, we describe the cloning of a novel *S. cerevisiae* gene *YPC1* that endows resistance to the fungal toxin fumonisin B1. Our data firmly establish that the yeast gene *YPC1* in fact encodes an alkaline ceramidase. First, YPC1p has an *in vitro* alkaline ceramidase activity with a pH optimum at pH 9.5. Second, YPC1p has substrate specificity toward yeast ceramides in that it catalyzes the breakdown of both phytoceramide and dihydroceramide but not mammalian unsaturated ceramide. Third, overexpression of YPC1p led to increased breakdown of labeled dihydroceramide and phytoceramide in intact yeast cells. Fourth, microsomes isolated from yeast cells that overexpress YPC1p had up to 30 times higher ceramidase activity than microsomes from cells that contain an empty vector. Finally, protein extracts from *E. coli* cells expressing YPC1p had significant increases in ceramidase activity as compared with extracts prepared from vector control bacterial cells. These data clearly indicate that YPC1 is indeed an alkaline ceramidase and not a regulator of the enzyme. We also demonstrate that YPC1p has the reverse activity that catalyzes the synthesis of yeast ceramides from palmitic acid and phytosphingosine or dihydrosphingosine. This ceramide synthase activity is coenzyme A-independent and occurs both in intact yeast cells and *in vitro*. This activity also explains why high copy expression of *YPC1* endowed resistance to fumonisin B1.

Several different ceramidase activities have been described from eukaryotic (9, 15) and prokaryotic systems (8). They are classified as acidic, neutral, and alkaline ceramidases based on their pH optimum. Acid ceramidase was initially described from rat brain (30). At that time it was reported to have reverse catalytic activity. The acidic ceramidase is the enzyme whose inherited deficiency underlies ceramide accumulation in the lysosomal storage disease known as Farber's disease (33). The enzyme was subsequently purified from human urine, cloned from human fibroblasts (11), and recently cloned from mouse tissue (12). It is localized in the lysosome, and it appears to prefer ceramides over dihydroceramides (11). However, there is no available evidence of the ability of the purified enzyme to catalyze the reverse reaction of ceramide synthase. Neutral ceramidase has been described from liver plasma membranes (34) and from rat intestinal brush border (35); however, not much is known about this enzyme. Recently, a membrane-bound ceramidase from rat brain was purified to homogeneity (15). It had an optimum pH in the neutral to alkaline range, appeared distinct from previously described ceramidases, and was termed non-lysosomal ceramidase. This enzyme also preferred unsaturated ceramide as substrate but was not evaluated for reverse catalytic activity. Alkaline ceramidase activity has been described from human cerebellum (31), fibroblasts (36, 37), and rat tissue (15, 34). In addition, two alkaline ceramidases were purified and characterized from guinea pig skin epidermis (32). Very recently an alkaline ceramidase was purified from the skin of patients with atopic dermatitis (8). This enzyme was from *Pseudomonas aeruginosa* that colonized skin; it required Ca<sup>2+</sup> for activity, and it hydrolyzed both ceramides and dihydroceramides equally but hydrolyzed phytoceramides less efficiently (8). Interestingly, this enzyme had the reverse activity of ceramide synthase. The *S. cerevisiae* alkaline ceramidase YPC1p appears to belong to this latter group of alkaline ceramidases. The ceramide synthase activity of YPC1p is distinguished from previously characterized ceramide synthesis activity by the following criteria. First, it is CoA-independent; second, it is not inhibited by fumonisin B1; and third, it prefers phytosphingosine and dihydrosphingosine.

YPC1p acts as a ceramidase or ceramide synthase, apparently depending on the availability of its substrate(s). In normally growing yeast cells, YPC1p overexpression led to the breakdown of phytoceramide or dihydroceramide. However, upon the addition of fumonisin B1 and consequent inhibition of endogenous yeast ceramide synthesis, YPC1p was able to catalyze the synthesis of phytoceramide from palmitic acid and phytosphingosine both in cells and *in vitro*. In both yeast and mammalian cells, the synthesis of ceramide is mainly catalyzed by ceramide synthase that uses long chain bases and fatty acyl-CoAs as substrates and is inhibited by fumonisin B1. Identification of YPC1p as a dual activity enzyme indicates that a yet uncharacterized salvage pathway to synthesize ceramides exists in yeast. Therefore, YPC1p has a potentially important role in the synthesis of ceramide, especially when the CoA-dependent ceramide synthase is disabled. Importantly, the ability of this enzyme to catalyze ceramide synthesis and its lack of sensitivity to fumonisin B1 raises the possibility that fumonisin B1 is unable to inhibit totally ceramide synthesis.

Additionally, we have identified a protein encoded by a human expressed sequence tag that has very high homology to YPC1p. This homologue is likely to have dual activity, thus lending support to the notion that this salvage pathway for ceramide synthesis also exists in mammalian cells.

Regulation of both ceramide levels in yeast and mammalian cells is critical and must be carefully executed. On the one hand, ceramide is an essential building block for sphingolipids, whereby sufficient ceramide is needed for cell growth and viability (38, 39). On the other hand, ceramide is a stress sensor regulating many cellular responses including heat stress (18), growth arrest (18), and cell death (40). In addition cellular levels of sphingosine (the product of ceramidase) and sphingosine phosphate are critical for cellular well being in that they may mediate proliferation and oppose ceramide-induced apoptosis (41). YPC1p and its mammalian homologue, therefore, may be critically poised to readjust sphingolipid flow in cells depending on cellular levels of sphingosines and ceramides.

Deletion of *YPC1* is viable and has no apparent phenotype but does affect endogenous metabolism of sphingolipids. As mentioned this gene has a homologue in *S. cerevisiae*. It remains to be evaluated if *YPC1* and its homologue have similar functions. It is possible that deletion of both genes will be required for phenotype studies to be meaningful.

Identification of YPC1p as an alkaline ceramidase has advanced our knowledge of sphingolipid metabolism in the yeast *S. cerevisiae*. It is the first sphingolipid hydrolase to be identified in yeast. This affirms our belief that sphingolipid metabolic pathways are conserved between *S. cerevisiae* and higher eukaryotic species. These studies also underscore the importance of yeast genetic approaches to clone novel enzymes of sphingolipid metabolism.

*Acknowledgments*—We thank Dr. Yusuf Hannun for critical review of the manuscript and helpful discussions, Dr. George Fam (Biotechnology Resource Laboratory at Medical University of South Carolina) for assistance with DNA sequencing, and Dr. Samer El Bawab for assistance with the assay of ceramidase activity.

#### REFERENCES

1. Hannun, Y. A. (1996) *Science* **274**, 1855–1859
2. Hannun, Y. A., and Obeid, L. M. (1997) *Biochem. Soc. Trans.* **25**, 1171–1175
3. Spiegel, S., Cuvillier, O., Edsall, L. C., Kohama, T., Menzeleev, R., Olah, Z., Olivera, A., Pirianov, G., Thomas, D. M., Tu, Z., Van Brocklyn, J. R., and Wang, F. (1998) *Ann. N. Y. Acad. Sci.* **845**, 11–18
4. Dickson, R. C., and Lester, L. R. (1999) *Biochim. Biophys. Acta* **1438**, 305–321
5. Merrill, A. H., Jr., and Wang, E. (1992) *Methods Enzymol.* **209**, 427–437
6. Wu, W. I., McDonough, V. M., Nickels, J. T., Jr., Ko, J., Fischl, A. S., Vales, T. R., Merrill, A. H., Jr., and Carman, G. M. (1995) *J. Biol. Chem.* **270**, 13171–13178
7. Ito, M., Kurita, T., and Kita, K. (1995) *J. Biol. Chem.* **270**, 24370–24374

8. Okino, N., Tani, M., Imayama, S., and Ito, M. (1998) *J. Biol. Chem.* **273**, 14368–14373
9. Hassler, D. F., and Bell, R. M. (1993) *Adv. Lipid Res.* **26**, 49–57
10. Bernardo, K., Hurwitz, R., Zenk, T., Desnick, R. J., Ferlinz, K., Schuchman, E. H., and Sandhoff, K. (1995) *J. Biol. Chem.* **270**, 11098–11102
11. Koch, J., Gartner, S., Li, C. M., Quintern, L. E., Bernardo, K., Levran, O., Schnabel, D., Desnick, R. J., Schuchman, E. H., and Sandhoff, K. (1996) *J. Biol. Chem.* **271**, 33110–33115
12. Li, C. M., Hong, S. B., Kopal, G., He, X., Linke, T., Hou, W. S., Koch, J., Gatt, S., Sandhoff, K., and Schuchman, E. H. (1998) *Genomics* **50**, 267–274
13. Nikolova-Karakashian, M., Morgan, E. T., Alexander, C., Liotta, D. C., and Merrill, A. H., Jr. (1997) *J. Biol. Chem.* **272**, 18718–18724
14. Coroneos, E., Martinez, M., McKenna, S., and Kester, M. (1995) *J. Biol. Chem.* **270**, 23305–23309
15. El Bawab, S., Bielawska, A., and Hannun, Y. A. (1999) *J. Biol. Chem.* **274**, 27948–27955
16. Saba, J. D., Nara, F., Bielawska, A., Garrett, S., and Hannun, Y. A. (1997) *J. Biol. Chem.* **272**, 26087–26090
17. Skrzypek, M. S., Nagiec, M. M., Lester, R. L., and Dickson, R. C. (1999) *J. Bacteriol.* **181**, 1134–1140
18. Mao, C., Saba, J. D., and Obeid, L. M. (1999) *Biochem. J.* **342**, 667–675
19. Rothstein, R. (1991) *Methods Enzymol.* **194**, 281–301
20. Mao, C., Wadleigh, M., Jenkins, G. M., Hannun, Y. A., and Obeid, L. M. (1997) *J. Biol. Chem.* **272**, 28690–28694
21. Gietz, R. D., and Sugino, A. (1988) *Gene (Amst.)* **74**, 527–534
22. Gietz, R. D., Schiestl, R. H. (1995) *Methods Mol. Cell Biol.* **5**, 255–269
23. Bielawska, A., Hannun, Y. A. (1999) *Methods Enzymol.* **311**, 499–518
24. Bielawska, A., Szulc, Z., and Hannun, Y. A. (1999) *Methods Enzymol.* **311**, 499–518
25. Mitsutake, S., Kita, K., Okino, N., and Ito, M. (1997) *Anal. Biochem.* **247**, 52–57
26. Mandala, S. M., Thornton, R. A., Frommer, B. R., Curotto, J. E., Rozdilsky, W., Kurtz, M. B., Giacobbe, R. A., Bills, G. F., Cabello, M. A., Martin, I., Peláez, F., and Harris, G. H. (1995) *J. Antibiot. (Tokyo)* **48**, 349–356
27. Bunting, M., Tang, W., Zimmerman, G. A., McIntyre, T. M., and Prescott, S. M. (1996) *J. Biol. Chem.* **271**, 10230–10236
28. Beeler, T., Bacikova, D., Gable, K., Hopkins, L., Johnson, C., Slife, H., and Dunn, T. (1998) *J. Biol. Chem.* **273**, 30688–30694
29. Haak, D., Gable, K., Beeler, T., and Dunn, T. (1997) *J. Biol. Chem.* **272**, 29704–29710
30. Gatt, S. (1966) *J. Biol. Chem.* **241**, 3724–3730
31. Sugita, M., Williams, M., Dulaney, J. T., and Moser, H. W. (1975) *Biochim. Biophys. Acta* **398**, 125–131
32. Yada, Y., Higuchi, K., and Imokawa, G. (1995) *J. Biol. Chem.* **270**, 12677–12684
33. Sugita, M., Dulaney, J. T., and Moser, H. W. (1972) *Science* **178**, 1100–1102
34. Spence, M. W., Beed, S., and Cook, H. W. (1986) *Biochem. Cell Biol.* **64**, 400–404
35. Hertervig, E., Nilsson, A., Nyberg, L., and Duan, R. D. (1997) *Cancer (Phila.)* **79**, 448–453
36. Chatelut, M., Feunteun, J., Harzer, K., Fensom, A. H., Basile, J. P., Salvayre, R., and Levade, T. (1996) *Clin. Chim. Acta* **245**, 61–71
37. Momoi, T., Ben-Yoseph, Y., and Nadler, H. L. (1982) *Biochem. J.* **205**, 419–425
38. Pinto, W. J., Wells, G. W., and Lester, R. L. (1992) *J. Bacteriol.* **174**, 2575–2581
39. Hanada, K., Hara, T., Fukasawa, M., Yamaji, A., Umeda, M., and Nishijima, M. (1998) *J. Biol. Chem.* **273**, 33787–33794
40. Obeid, L. M., Linardic, C. M., Karolak, L. A., and Hannun, Y. A. (1993) *Science* **259**, 1769–1771
41. Cuvillier, O., Pirianov, G., Kleuser, B., Vanek, P. G., Coso, O. A., Gutkind, S., and Spiegel, S. (1996) *Nature* **381**, 800–803