The Transcriptional Response of Saccharomyces cerevisiae to Pichia membranifaciens Killer Toxin

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The transcriptional response of Saccharomyces cerevisiae to Pichia membranifaciens killer toxin (PMKT) was investigated. We explored the global gene expression responses of the yeast S. cerevisiae to PMKT using DNA microarrays, real time quantitative PCR, and Northern blot. We identified 146 genes whose expression was significantly altered in response to PMKT in a non-random functional distribution. The majority of induced genes, most of them related to the high osmolarity glycerol (HOG) pathway, were core environmental stress response genes, showing that the coordinated transcriptional response to PMKT is related to changes in osmotic homeostasis. Hog1p was observed to be phosphorylated in response to PMKT implicating the HOG signaling pathway. Individually deleted mutants of both up- (99) and down-regulated genes (47) were studied for altered sensitivity; it was observed that the deletion of up-regulated genes generated hypersensitivity (82%) to PMKT. Deletion of down-regulated genes generated wild-type (36%), resistant (47%), and hypersensitive (17%) phenotypes. This is the first study that shows the existence of a transcriptional response to the poisoning effects of a killer toxin.

Killer phenomena are widespread in yeasts. Killer toxins are proteins or glycoproteins that are lethal to sensitive strains of the same species and a different variety of other yeast genera. In this line, attention has focused mainly on the characterization of killer toxins from Saccharomyces cerevisiae (K1, K2, and K28) followed more recently by the investigation of yeasts such as Kluyveromyces lactis, Zygosaccharomyces bailii, Hanseniaspora uvarum, Pichia membranifaciens, Debaryomyces hansenii, Schwanniomyces occidentalis, etc. (1–7).

P. membranifaciens CYC 1106 is a strain originally isolated from fermenting olive brines with pronounced killer activity against a variety of yeast species (8) and fungi (9). Once the protein nature of the toxin produced was established, the secreted protein was purified from the supernatant of growing cultures of P. membranifaciens. Previous biochemical studies on the PMKT1 mechanism of killing of sensitive yeast cells indicated that PMKT is an 18-kDa protein that interacts with the (1 → 6)-β-D-glucans of the cell wall of sensitive yeasts (6, 10). Recently the killing mechanism of this killer toxin has been elucidated (10). Regardless of certain possible additional effects, the killer toxin of P. membranifaciens CYC 1106 acts by disrupting plasma membrane electrochemical gradients. The death of sensitive cells in the presence of killer toxin is characterized by a leakage of common physiological ions through non-regulated ion channels in the plasma membrane causing a discharge of cellular membrane potential and changes in ionic homeostasis in a way comparable to that of certain killer toxins (K1) (11). Non-selective channel formation has been suggested to be the cytotoxic mechanism of action of PMKT (10).

Yeasts must cope with different adverse environmental conditions, including heat shock, oxidative stress, high osmolarity, extreme pH values, nutrient availability, and toxins from plants, fungi, or bacteria as well as heavy metals and different xenobiotics. Yeasts have therefore adapted to growth under these conditions by developing a variety of protective mechanisms ranging from general stress responses to highly specific regulatory pathways. A variety of changes in the environment activate multiple mitogen-activated protein kinase cascades, which convert these signals into appropriate metabolic responses (12, 13). Five mitogen-activated protein kinase cascades that coordinate intracellular activities in response to rapidly changing environmental conditions have been characterized (14). Because of our knowledge about its sequenced genome and the availability of powerful genetic tools, S. cerevisiae has been a particularly valuable model system for studying such responses. Of particular significance for this unicellular eukaryote is its response to changes in the osmolarity of the medium. Yeast cells are able to detect and respond to changes in osmolarity by two independent osmosensors: Sln1p and Sho1p (15–17). These membrane-bound proteins activate the so-called high osmolarity glycerol (HOG) mitogen-activated protein kinase pathway, and this results in a rise in the intracellular glycerol concentration to adapt the cellular osmotic pressure and to prevent the loss of water (18–21). These survival mechanisms must operate within the first seconds after a sudden osmotic shift because water loss or uptake occurs very fast (22). The stimulation of glycerol synthesis is achieved by increasing the transcription of genes such as GPD1 (encoding glycerol-3-phosphate dehydrogenase), GPP2 (glycerol-3-phosphate phosphatase), HSP12 (heat shock protein), CTTI (cytosolic catalase T), YAP4 (yeast activator protein), STL1 (encoding a member of the hexose transporter family of the major facilitator superfamily), etc. (23–33). Regardless of their effects on osmolarity, high concentrations of certain ions, such as lithium or sodium, may be toxic for yeast cells because they block several metabolic reactions (34). In this case, the transcriptional activation of the ENA1 gene, which encodes a P-type ATPase, is required for the efflux of sodium ions (35, 36). Two plasma membrane proteins, Psr1p and Psr2p, are essential for an efficient sodium ion stress response through transcriptional activation of ENA1 (37). Apart from the HOG-dependent induction during sodium ion stress, ENA1 transcription is also induced by another independent pathway mediated by the calcium/calmodulin-dependent phosphatase calcineurin (38–40).

The aim of this study was to determine the global gene expression responses of S. cerevisiae to the killer toxin produced by P. membranifaciens CYC 1106 with a view to gaining insight into the mechanisms and processes underlying the killing of sensitive yeast cells. Here for the...
**The Transcriptional Response to PMKT**

first time we report evidence that the transcriptional response of *S. cerevisiae* to the presence of PMKT is very similar to the response of cells undergoing adaptation to ionic or osmotic changes in the cellular environment. Western analysis of the signaling through the HOG pathway revealed a Hog1p phosphorylation in response to PMKT. The analysis of arrayed ORFs was complemented and extended by the fact that a high proportion of deletion mutants of genes whose expression was observed to be affected by PMKT had altered sensitivity phenotypes.

**EXPERIMENTAL PROCEDURES**

*Yeast Strains and General Media—*The killer strain used in this study was *P. membranifaciens* CYC 1106 (Complutense Yeast Collection, Complutense University of Madrid, Madrid, Spain) originally isolated from olive brines (41). The sensitive wild-type strain used in this study was *S. cerevisiae* Hansen BY4743 (MATa/MATa his3Δ1/Δ1 his3Δ1 leu2Δ0/Δ0 life2Δ0 lys2Δ0 MET15/MET15 ura3Δ0/Δ0) (deletion parental strains, catalog number 95400.BY4743, Invitrogen). Deletant strains were from the Saccharomyces Genome Deletion Project and are available commercially at Invitrogen (Yeast Deletion Pools-Homozygous Diploid, catalog number 95401.H1Pool). Homozygous diploids mutants were BY4743 (orf Δ::kan MX4/orf Δ::kan MX4).

The basic medium used for this study was YMA medium (1% (w/v) glucose, 0.3% (w/v) yeast extract (Difco), 0.3% (w/v) malt extract (Difco), 0.5% (w/v) protease peptone Number 3 (Difco), and 2% agar). The strains were maintained at 20 °C in YMA medium supplemented with 15% glycerol, 200 mg/liter G418 (Geneticin), and 2% agar. Time course experiments of the killing process were carried out in buffered YMB medium (YMA medium without glycerol, Geneticin, and agar) and buffered with 100 mM sodium citrate-phosphate, pH 4.0. Killer toxin activity was determined on YMA-MB agar plates (YMA medium (without olive glycerol and Geneticin) supplemented with 30 mg/liter methylene blue and 100 mM sodium citrate-phosphate buffer, pH 4.0).

**Killer Toxin Assay—**We assayed for killer toxin sensitivity with a diffusion test using 6-mm-diameter antibiotic assay AA Whatman paper discs on buffered YMA-MB seeded with the selected *S. cerevisiae* mutant strains in parallel with the wild-type strain. Incubation was carried out at 20 °C because killer factor is rapidly inactivated at temperatures above 25 °C. The diameter of the inhibition zone was used as a measure of sensitivity to the killer toxin. For each mutant, sensitivity was tested up to three times, and the results were compared with those from the wild-type strain (mutant inhibition area)/(wild-type inhibition area) × 100).

**Purification Procedure—** *P. membranifaciens* CYC 1106 was cultured in YNB-D-Brij 58 medium (yeast nitrogen base-dextrose; Difco), 3 × 1 liter, in 2-liter Erlenmeyer flasks for 3 days at 20 °C at 150 rpm. The cells were centrifuged (4,000 × g for 10 min at 4 °C), and the supernatant was adjusted to a final glycerol concentration of 15% (v/v). The purification process was done as reported previously (9).

**Measurement of Cell Death—** *S. cerevisiae* BY4743 cells were grown to logarithmic phase in buffered YMB medium, collected, and subsequently resuspended in the same medium containing killer activity (1,205 AU/ml). The final cell concentration was 10⁸ cells/ml. A control with heat-inactivated (5 min at 75 °C) killer toxin was run in parallel. Aliquots were taken periodically, and additional 10-fold dilutions were made serially to a final dilution of 10⁻⁶. Four volumes of 50 μl each were used for plating on YMA medium. The colonies were counted after 48 h of growth at 30 °C.

**Experimental Design and RNA Isolation—** For RNA isolation, time course experiments of the killing process, using *S. cerevisiae* BY4743, were performed as three independent biological repeats. Approximately 200 ml of asynchronously grown cells were cultured in YMB medium (buffered with 100 mM sodium citrate-phosphate, pH 4.0) at 20 °C, shaking at 125 rpm until an A₆₀₀ of 0.5 was reached. Ten milliliters of cells were harvested by centrifugation (14,000 rpm for 1 min at 4 °C) and snap-frozen in liquid nitrogen (at this point cells were collected for determination of expression profiles under basal conditions). Immediately afterward the rest of the culture was exposed to the killer toxin (1,205 AU/ml), and samples of 10 ml each were collected at 0, 15, 30, 45, 60, 90, and 120 min. Then samples for RNA extraction were harvested and frozen as above. Total RNA was extracted using the hot acid phenol method (42).

**Microarray Hybridization, Scanning, and Data Acquisition—** Based on the results on cell death, measured by plating, reverse transcription (Superscript, Invitrogen) was performed for 40 μg using total RNA extracted from cells after 45 min of exposure to PMKT. After the Cy3- and Cy5-dCTP-labeled cDNAs had been hybridized onto glass DNA microarrays containing 100% of all known and predicted *S. cerevisiae* genes, 40 μl of the mixed cDNA solution were allowed to hybridize to the microarrays at 62 °C for 12 h (Agilent hybridization chamber, Agilent Technologies, Palo Alto, CA). When the hybridization process was completed, the microarrays were washed with buffer A (0.1% SDS, 0.6× SSC) at 50 °C followed by a 5-min wash in buffer B (0.03× SSC) at 20 °C. Microarrays were scanned with Agilent scanner G2565BA (Agilent Technologies), and microarray images were analyzed with Agilent Feature Extraction software, version 7.5 (43, 44). If expression ratios were > +3.0 (up-regulated) or < −2.0 (down-regulated) in four different arrays, the corresponding genes were then considered to be expressed differentially, and the average ratio from the change in expression was calculated and treated as the true ratio between the different arrays.

Only genes with constant expression ratios between different experiments were considered. It must be taken into account that a large proportion of the genes (those between +3.0-fold induction and −2.0-fold repression) responded, according to our results, in an apparently random manner. It is known that when a stimulus occurs the nature of regulated transcripts changes over time, suggesting that different functions need to be activated at different time points. The origin of this variability is unknown but may be due to events that affect cells at different moments of the Killing process but not simultaneously for the whole PMKT-treated yeast population. Other possibilities are small differences in toxin purity (high performance liquid chromatography-tested), media composition, or cell age. In light of this, we decided to confirm these results with quantitative real time PCR under the assumption that the constantly expressed genes were the main ones involved in the underlying process of killing.

**Real Time Quantitative PCR—** Total RNA was obtained from cells at a time point of 45 min and then treated with DNase I (Sigma). cDNA was synthesized from samples of 1.5 μg of total RNA making use of the random primer procedure and the RETRoscript kit (Ambion, Austin, TX) following the manufacturer’s instructions. Q-PCR was performed on an ABI PRISM® 7900 HT (Applied Biosystems, Warrington, UK) device using the SYBR Green PCR Master Mix (Applied Biosystems) and specific oligonucleotide primers at a concentration of 300 nM. Matching oligonucleotide primers were designed using the Primer Express software (PerkinElmer-Applied Biosystems) and Oligo software (Oligo Analyzer) and synthesized by Sigma-Genosys (Sigma-Genosys, Ltd). Amplification of cDNA was performed over 40 cycles. The first cycle was performed at 95 °C for 10 min. Cycles 2–40 were performed at 95 °C for 15 s followed by 60 °C for 1 min. Each assay was performed in quadruplicate. For normalization of cDNA loading, all samples were run in parallel with a housekeeping gene (IPP1, inorganic
pyrophosphatase). The specificity of PCR amplifications from the different sets of oligonucleotide primers was examined routinely by agarose gel electrophoresis.

Northern Blot Analysis of the PMKT-dependent Genes—According to the results from microarrays, confirmed by Q-PCR, some osmorepressive genes (GPD1 and GPP2) as well as CTTI and HSP12, two general stress-responsive genes; PDR12 and TRK2, two genes highly induced at low pH; and ENA1, a gene known to be induced under high salt conditions, were studied by Northern blotting (32). To determine the extent of induction or repression of gene expression, all signals were compared with IPP1, which encodes inorganic pyrophosphatase, whose expression is not affected by osmotic stress (33). Total RNA was isolated from cells (BY4743 and hog1Δ) at the selected time points (0, 15, 30, 45, 60, 90, and 120 min), separated by formaldehyde gels, and transferred onto nylon membranes (Hybond XL, Amersham Biosciences). Probes were generated by PCR from chromosomal DNA of BY4743. PCR probes of GPD1, GPP2, CTII, HSP12, PDR12, TRK2, ENA1, and IPP1 were radio-labeled by random priming with [α-32P]ATP (MegaPrime, Amersham Biosciences). G-50-purified, and hybridized overnight at 65 °C in hybridization buffer (7% (w/v) SDS, 0.25M sodium phosphate buffer, pH 7.5, 1 mM EDTA, and 1% (w/v) bovine serum albumin). The blots were washed (20 mM sodium phosphate, pH 7.5, 0.1% (w/v) SDS, and 1 mM EDTA), and the signal was detected either by exposure to radioisensitive film (Biomax MR, Eastman Kodak Co.). Values were normalized by comparison with IPP1 signals.

Western Blotting of Hog1p and Hog1p Phosphorylation—Separation of total soluble protein (30 µg of total protein), isolated as described previously (45, 46), was accomplished on 12% polyacrylamide gels (Bio-Rad), whereas subsequent Western blotting of Hog1p and phosphorylated Hog1p was performed according to standard procedures on polyvinylidene difluoride membranes (Bio-Rad). Briefly total soluble protein was isolated from S. cerevisiae BY4743 strain grown in buffered YMB medium after 0, 5, 10, 15, 30, 45, and 60 min of exposure to PMKT. Dual phosphorylation of Hog1p was determined using an anti-dually phosphorylated (Thr-174 and Tyr-176) p38 antibody (New England Biolabs, Beverly, MA). Hog1p was detected using an anti-C-terminal Hog1p antibody (Yc20, catalog number sc-6815, Santa Cruz Biotechnology, Beverly, MA). Hog1p phosphorylation is not affected by osmotic stress (33). Total RNA was isolated from cultures grown for glycerol and dry weight determinations. Intracellular glycerol was measured by drying filters with cells at 80 °C for 16 h. To determine the glycerol concentration of the extracellular medium during PMKT treatment, 1-mL samples of the treated cultures were centrifuged, and the supernatant was used for glycerol assays. Glycerol was determined with a glycerol measurement kit (catalog number 148270, Roche Applied Science).

RESULTS

Genome-wide Gene Expression Profiles—To examine the gene expression response to PMKT a time course study of the killing process was carried out. After exposure of asynchronously grown cells to PMKT at times between 0 and 8 h (not shown), cell death was observed to begin after 1 h. The cell death rate in the presence of PMKT (1,205 AU/ml) was 0.22 h−1. From these death kinetics, a PMKT dosage of 1,205 AU/ml and sampling times of 0 min (untreated control) and 45 min (treated cells) after exposure to PMKT were chosen for mRNA isolation and subsequent microarray studies and Q-PCR.

Embryonic gene expression profiles of S. cerevisiae cultures grown asynchronously were examined after exposure to PMKT using whole-genome microarrays and Q-PCR. Similar results were observed for mRNA expression levels using both techniques. Validation of microarray results with Q-PCR was done to verify that array data were not the result of problems inherent to the array technology. The grouped distribution of genes that were either up- or down-regulated after exposure to PMKT (TABLE ONE and TWO) provided information pertinent to the killing mechanism of PMKT and to the response of S. cerevisiae to the toxin at the molecular level. The mRNA level of a total of 99 genes was at least 3-fold higher after the addition of PMKT (TABLE ONE). Forty-seven genes were observed to exhibit 2-fold or higher repression levels after 45 min of exposure to PMKT (TABLE TWO).

In our results, the functional group of up-regulated genes with the highest representation (31 genes) was the group of genes related to signal transduction, gene expression, transcription, and RNA processing, showing that there is an important underlying cellular response to PMKT. In this group of genes TF51, NAM8, YAPI, KQKO, SCH9, STE20, PBS2, MED2, SRB4, HAL9, and GAL11 had the highest expression levels. Between them, YAPI, XBP1, SCH9, and HAL9 were directly related to cell stress or salt tolerance.

During the response to PMKT, many induced ORFs were observed in the category of ionic homeostasis and transport facilitation (TABLE ONE) (35, 47, 48). Among them several transcripts were up-regulated (ENA1/PM2, NHA1, IST1, TOK1, PS51, PHO84, PHO89, PHO87, ZRT1, and CTR3).

Indeed the dehydrogenases and phosphatases leading to glycerol production, GPD1 and GPP2, were up-regulated. Similarly ORFs for enzymes involved in trehalose metabolism, GLK1, PGM2, TSL1, TPS1, and NTH1, were up-regulated during PMKT activity.

Heat shock proteins (HSPs) were found to be up-regulated in response to PMKT (HSP12, HSP104, HSP82, HSP26, DDR2, SSA2, SSA2, and SSA1). Furthermore we found a severe induction of CTII expression in the presence of PMKT, showing that a general stress response occurred in the presence of the toxin (TABLE ONE and Fig. 1). PMKT also induced YAPI, which specifically mediates oxidative stress responses and appears to be responsible for the rapid establishment of a transient transcriptional response.

Northern Blots—Subsequent time course studies of the expression of some induced genes using Northern blot (Fig. 1) revealed that the process of killing occurred after the shift in mRNA synthesis had taken place, showing that changes in mRNA levels in response to PMKT occurred 30–45 min before the process of killing could be observed quantitatively by plating (not shown). Then mRNA levels did not decrease with time to restore the initial expression levels in a process probably related to cell death (with cells unable to regulate their mRNA levels) or to a permanent stimulation of the remaining active cells. The hog1Δ mutant had a similar response to PMKT, but the amount of mRNA of many of the induced genes were severalfold lower in comparison with the wild-type strain (ENA1, 18-fold lower; GPP2, 9-fold; TRK2, GPD1, and HSP12, 5-fold; CTII, 4-fold; and PDR12, 2.5-fold).

Glycerol Production and Leakage—Our results showed that treatment with PMKT induces glycerol synthesis, and this process was indeed observed in the first 30 min after toxin addition. The same result was observed in the hog1Δ mutant, which is hypersensitive to PMKT,
### TABLE ONE

**Genes induced by more than 3-fold after a PMKT exposure**

| Gene name | ORF     | Description of gene product                                                                 | -Fold induction | PMKT death zone |
|-----------|---------|--------------------------------------------------------------------------------------------|-----------------|-----------------|
|           |         |                                                                                             | Microarrays     | Q-RT-PCR        |
| Signal transduction, gene expression, transcription, and RNA processing (31 genes) |         |                                                                                             |                 |                 |
| TFS1      | YLR178C | Cdc25-dependent nutrient and ammonia response cell cycle regulator                           | 15.3            | 12.5            | 108             |
| GSP2      | YOR185C | GTP-binding protein involved in trafficking through nuclear pores                            | 5.1             | 4.3             | 104             |
| NAM8      | YHR086W | Protein involved in meiotic recombination                                                   | 10.2            | 10.1            | 106             |
| GIP2      | YER054C | Glc7p-interacting protein                                                                    | 4.3             | 3.8             | 105             |
| SDS22     | YKL193C | Regulatory subunit for the mitotic function of type I protein phosphatase                   | 5.5             | 4.8             | 106             |
| YAPI*     | YML007W | Transcription factor involved in halotolerance and oxidative stress response               | 12.7            | 11.6            | 121             |
| CUP2      | YGL166W | Copper-dependent transcription factor                                                         | 6.6             | 7.2             | 103             |
| XBP1*     | YIL101C | Stress-induced transcriptional repressor                                                    | 3.8             | 3.6             | WT              |
| REG1      | YDR028C | Regulator subunit for protein phosphatase Glc7p                                             | 4.1             | 4.1             | 102             |
| PAPI      | YKR002W | Poly(A) polymerase required for mRNA 3’ end formation                                       | 4.0             | 3.9             | 108             |
| KSP1      | YHR082C | Serine/threonine protein kinase                                                              | 5.2             | 4.3             | 114             |
| KKQ8      | YKL168C | Weak similarity to serine/threonine protein kinase                                           | 14.1            | 11.7            | 109             |
| PTK2      | YJR059W | Serine/threonine protein kinase of required for polyamine uptake                           | 8.1             | 6.4             | 104             |
| AKL1      | YBR059C | Ark family kinase-like protein; probable serine/threonine-specific protein kinase           | 3.5             | 3.3             | 110             |
| SCH9*     | YHR205W | Serine/threonine protein kinase involved in stress response and nutrient-sensing signaling pathway | 10.6           | 12.5            | 138             |
| STE20     | YHL007C | Serine/threonine protein kinase of the pheromone pathway                                     | 15.9            | 13.8            | 114             |
| PSK1*     | YAL017W | PAS kinase involved in the control of sugar metabolism and translation                      | 8.5             | 10.1            | 123             |
| PBS2      | YIL128C | Tyrosine protein kinase of the MAP kinase kinase family                                     | 19.9            | 18.5            | 125             |
| MED2      | YDL005C | Transcriptional regulation mediator                                                          | 15.3            | 14.5            | 103             |
| ITC1      | YGL133W | Subunit of lsw2 chromatin-remodeling complex                                                 | 6.6             | 8.4             | 107             |
| SW16      | YLR182W | Transcription factor                                                                         | 3.3             | 2.3             | 103             |
| TIS11     | YLR136C | tRNA-specific adenosine deaminase 3                                                         | 7.1             | 5.3             | WT              |
| UGA3      | YDL170W | Transcriptional activator for GABA catabolic genes                                           | 3.2             | 4.3             | 104             |
| SRB2      | YHR041C | DNA-directed RNA polymerase II holoenzyme and Kornberg’s mediator (SRB) subcomplex subunit | 5.3             | 5.3             | WT              |
| SRB4      | YER022W | DNA-directed RNA polymerase II holoenzyme and Kornberg’s mediator (SRB) subcomplex subunit | 14.2            | 17.7            | 101             |
| GAL11     | YOL051W | DNA-directed RNA polymerase II holoenzyme and Kornberg’s mediator (SRB) subcomplex subunit | 12.7            | 11.5            | 104             |
| HAL1*     | YOL089C | Transcriptional activator of ENA1                                                           | 9.2             | 9.8             | 124             |
| PRP18     | YGR006W | U5 snRNA-associated protein                                                                  | 8.2             | 7.7             | 110             |
| NRS1      | YGR159C | Nuclear localization sequence-binding protein                                               | 3.4             | 3.5             | 101             |
| CDC40     | YDR364C | Required for mRNA splicing                                                                   | 3.1             | 3.7             | 109             |
| CBC2      | YPL178W | Small subunit of the nuclear cap-binding protein complex CBC                                 | 5.5             | 6.6             | 103             |
| Glycerol, trehalose, and glycogen (11 genes) |         |                                                                                             |                 |                 |
| TPS1      | YBR126C | Trehalose-6-phosphate synthase; 56-kDa subunit/trehalose production                          | 11.4            | 10.8            | 104             |
| TSL1      | YML100W | α,β-trehalose-phosphate synthase, 123-kDa subunit                                           | 4.8             | 3.9             | 106             |
| NTH1      | YDR001C | Neutral trehalase (α,β-trehalase)/trehalose breakdown                                         | 3.0             | 2.9             | 106             |
| HOR2      | YER062C | Glyceraldehyde synthase/isofrom 1/glycerol production                                        | 5.2             | 5.4             | 108             |
| GPP1      | YIL053W | Glyceraldehyde 3-phosphate synthase/isofrom 2/glycerol production                           | 5.5             | 5.2             | 121             |
| GPP2      | YER063C | Glyceraldehyde 3-phosphate synthase/isofrom 2/glycerol production                           | 33.1            | 40.2            | 166             |
| Gene name | ORF | Description of gene product                                                                 | -Fold induction | PMKT death zone |
|-----------|-----|----------------------------------------------------------------------------------------------|-----------------|-----------------|
| GPD1      | YDL022W | Glycerol-3-phosphate dehydrogenase (NAD)/glycerol production                               | 41.1            | 154             |
| DAK1*     | YML070W | Dihydroxyacetone kinase, induced in high salt                                                  | 8.2             | 121             |
| GLC3      | YEL011W | α-1,4-Glucan branching enzyme/glycogen production                                              | 3.1             | 102             |
| MBR1      | YDL022W | Required for optimal growth on glycerol                                                        | 5.4             | 122             |
| FPS1      | YLL043W | Glycerol channel of the plasma membrane/also involved in arsenite and antimonite uptake (MIP family) | 16.3            | 109             |
| PPD1      | YDL013W | Hexose metabolism-related protein                                                              | 4.1             | 111             |
| PGM2      | YMR105C | Phosphoglucomutase, major isoform                                                              | 3.9             | 101             |
| INO1      | YCL153C | myo-Inositol-1-phosphate synthase                                                               | 5.3             | 103             |
| GLK1      | YCL040W | Aldohexose-specific glucokinase/glycolysis                                                      | 7.7             | 109             |
| TK12      | YBR117C | Transketolase isoform 2                                                                       | 5.2             | 113             |
| FUN50a    | YAL061W | High similarity to alcohol/sorbitol dehydrogenase                                              | 4.3             | 106             |
| HXT1      | YHR094C | Low affinity hexose facilitator                                                                | 3.5             | WT              |
| HXT11     | YOL156W | Hxt family protein with intrinsic hexose transport activity, unknown physiological function  | 6.9             | 102             |
| HXT5      | YHR096C | Hxt family protein with intrinsic hexose transport activity/sugar uptake                       | 10.1            | 133             |
| HXT14     | YNL318C | Protein of the sugar transporter superfamily, able to sustain slow growth on galactose        | 5.8             | 112             |
| STF1      | YDR536W | Member of the hox transporter family/sugar uptake                                               | 22.2            | 132             |
| GLOI*     | YML004C | Glyoxalase I                                                                                  | 9.3             | 108             |
| PSK1*     | YAL017W | PAS kinase involved in the control of sugar metabolism and translation                         | 8.5             | 123             |
| DLD3      | YEL071W | α-Lactate dehydrogenase                                                                        | 3.0             | 105             |
| ALD5      | YER073W | Aldehyde dehydrogenase (NAD), mitochondrial                                                   | 6.3             | 105             |
| ALD4      | YOR374W | Aldehyde dehydrogenase, mitochondrial                                                        | 17.9            | 116             |
| ALD2      | YMR170C | Aldehyde dehydrogenase (NAD and NADP), mitochondrial                                          | 19.3            | 111             |
| ALD3      | YER073W | Aldehyde dehydrogenase (NAD and NADP), cytosolic                                               | 18.4            | 112             |
| STF2      | YGR008C | ATPase stabilizing factor                                                                     | 4.6             | 112             |
| CYB2      | YML054C | Lactate dehydrogenase cytochrome b2                                                            | 11.3            | 105             |
| CYC7      | YEL039C | Cytochrome c isoform 2                                                                        | 3.1             | 104             |
| GRE3      | YHR104W | Aldose reductase (NADP)                                                                        | 6.6             | 101             |
| GCN4      | YEL009C | Transcriptional activator of amino acid biosynthetic genes                                    | 3.4             | 104             |
| AVT6      | YER119C | Involved in amino acid efflux from the vacuole                                                  | 5.1             | WT              |
| ARG9      | YHR137W | Aromatic amino acid aminotransferase II                                                       | 5.5             | WT              |
| PHO11     | YAR071W | Secreted acid phosphatase                                                                       | 8.2             | 110             |
| PHO5      | YBR093C | Repressible acid phosphatase precursor                                                         | 7.3             | 103             |
| PHO86a    | YJL117W | Targeting and packing of Pho84p inorganic phosphate transporter                                 | 3.9             | 103             |
| PHO84a    | YML123C | Inorganic phosphate permease                                                                   | 4.2             | 112             |
| PSRTa     | YLL010C | Plasma membrane phosphate involved in sodium stress response                                   | 7.9             | 127             |
| SDPI*     | YIL113W | Stress-inducible dual specificity phosphatase                                                    | 4.9             | 117             |
| PHO89*    | YBR296C | Na^+ coupled phosphate transport protein; high affinity                                         | 19.9            | 115             |
| PHO87a    | YCR037C | Low affinity phosphate transporter                                                              | 4.6             | 119             |

Ionic homeostasis and transport facilitation (19 genes)

| Gene name | ORF | Description of gene product | -Fold induction | PMKT death zone |
|-----------|-----|------------------------------|-----------------|-----------------|
| IST1      | YNL265C | Similarity to Nuf1p, has a role in resistance to high concentrations of sodium | 5.6 | 154 |
| NHAI      | YLR138W | Na^+/H^+ exchanger (also harboring K^+/H^+ activity) | 9.8 | 134 |
### TABLE ONE—CONTINUED

| Gene name | ORF   | Description of gene product                                                                 | -Fold induction | PMKT death zone |
|-----------|-------|----------------------------------------------------------------------------------------------|-----------------|-----------------|
|                 |       |                                                                                              | Microarrays     | Q-RT-PCR        |      |
| TOK1       | YIL093C | Voltage-gated, outward rectifying K⁺ channel protein of the plasma membrane                  | 3.3             | 4.9             | 108  |
| HAL9*      | YOL089C | Transcriptional activator of ENA1                                                            | 9.2             | 9.8             | 124  |
| ENA1       | YDR040C | P-type ATPase involved in sodium and lithium efflux, required for sodium tolerance           | 27.2            | 30.4            | 159  |
| SAT2       | YBR070C | Osmotolerance protein/probable membrane protein                                               | 8.8             | 7.8             | 121  |
| TRK2       | YKR050W | Potassium transporter II                                                                      | 25.1            | 22.8            | 123  |
| PSR1*      | YLL010C | Plasma membrane phosphatase involved in sodium stress response                              | 7.9             | 8.8             | 127  |
| PH086*     | YL117W  | Targeting and packing of Pho84p inorganic phosphate transporter                               | 3.9             | 4.0             | 103  |
| PH084*     | YML123C | Inorganic phosphate permease                                                                 | 4.2             | 4.3             | 112  |
| PH089*     | YBR296C | Na⁺-coupled phosphate transport protein; high affinity                                       | 19.9            | 20.0            | 115  |
| PH087*     | YCR037C | Low affinity phosphate transporter                                                             | 4.6             | 3.9             | 119  |
| GRE1*      | YCR223C | Induced by osmotic stress/hypothetical protein                                               | 6.1             | 5.7             | 113  |
| GIT1       | YCR099C | Glycrophosphoinositol transporter also able to mediate low affinity phosphate transport        | 4.6             | 4.7             | 105  |
| ZRT1       | YGL255W | High affinity zinc transport protein                                                           | 8.9             | 6.9             | 109  |
| SIT1       | YEL065W | Transporter of the bacterial siderophore ferrioxamine B/probably multidrug resistance protein | 6.6             | 6.3             | 97   |
| PDR12      | YPL058C | ABC transporter of the plasma membrane acting as a weak organic acid extrusion pump           | 20.9            | 20.8            | 133  |
| PDR5       | YOR153W | Pleiotropic drug resistance protein                                                            | 4.5             | 3.7             | 102  |
| CTR3       | YLR411W | Copper transport protein                                                                      | 4.1             | 3.9             | 106  |

**Cell stress and rescue (16 genes)**

| Gene name | ORF   | Description of gene product                                                                 | -Fold induction | PMKT death zone |
|-----------|-------|----------------------------------------------------------------------------------------------|-----------------|-----------------|
|           |       |                                                                                              | Microarrays     | Q-RT-PCR        |      |
| HSP12     | YFL014W | Heat shock protein                                                                           | 57.8            | 56.1            | 141  |
| HSP104    | YLL026W | Heat shock protein                                                                           | 8.8             | 5.9             | 111  |
| HSP82     | YPL240C | Heat shock protein                                                                           | 4.4             | 6.0             | 114  |
| HSP26     | YBR072W | Heat shock protein                                                                           | 13.5            | 12.4            | 119  |
| DDR2      | YOL052C-A | Heat shock protein DDRA2                                                                      | 16.6            | 13.4            | 122  |
| SSA2      | YLL024C | Heat shock protein of HSP70 family, cytosolic                                                | 4.6             | 5.6             | 105  |
| SSA1      | YNL209W | Heat shock protein of HSP70 family, cytosolic                                                | 5.1             | 4.6             | 101  |
| FAP7*     | YDL166C | Involved in the oxidative stress response                                                    | 5.2             | 6.3             | 114  |
| WSC4      | YHL028W | Cell wall integrity and stress response component 4                                         | 4.9             | 3.5             | 102  |
| SCHF9*    | YHR205W | Serine/threonine protein kinase involved in stress response and nutrient-sensing signaling pathway | 10.6            | 12.5            | 138  |
| CTT1*     | YGR088W | Catalase T, cytosolic                                                                        | 37.7            | 44.2            | 125  |
| XB1*      | YIL101C | Stress-induced transcriptional repressor                                                     | 3.8             | 3.6             | WT   |
| STI1      | YOR027W | Stress-induced protein                                                                       | 5.6             | 5.7             | 111  |
| PSR1*     | YLL010C | Plasma membrane phosphatase involved in sodium stress response                              | 7.9             | 8.8             | 127  |
| SDP1*     | YIL113W | Stress-inducible dual specificity phosphatase                                                | 4.9             | 5.8             | 117  |

**Protection against oxidative and chemical damage (eight genes)**

| Gene name | ORF   | Description of gene product                                                                 | -Fold induction | PMKT death zone |
|-----------|-------|----------------------------------------------------------------------------------------------|-----------------|-----------------|
|           |       |                                                                                              | Microarrays     | Q-RT-PCR        |      |
| GLO1*     | YML004C | Glyoxalase I                                                                                 | 9.3             | 10.0            | 108  |
| CTT1*     | YGR088W | Catalase T, cytosolic                                                                        | 37.7            | 44.2            | 125  |
| DAK1*     | YML070W | Dihydroxyacetone kinase, induced in high salt                                               | 8.2             | 7.7             | 121  |
| GRE2      | YOL151W | Similarity to plant dihydrofolavonol-4-reductases                                            | 5.5             | 5.4             | 109  |
| MPH1      | YIR002C | Protection of the genome from spontaneous and chemically induced damage/probable RNA helicase | 6.3             | 6.9             | WT   |
| YAPI*     | YML007W | Transcription factor involved in halotolerance and oxidative stress response                | 12.7            | 11.6            | 121  |
| FAP7*     | YDL166C | Involved in the oxidative stress response                                                   | 5.2             | 6.3             | 114  |
| CSF1      | YLR087C | Required for normal growth rate and resistance to NaCl and H₂O₂                              | 6.6             | 5.7             | 127  |

**Cell surface (two genes)**

| Gene name | ORF   | Description of gene product                                                                 | -Fold induction | PMKT death zone |
|-----------|-------|----------------------------------------------------------------------------------------------|-----------------|-----------------|
|           |       |                                                                                              | Microarrays     | Q-RT-PCR        |      |
| SPS100    | YHR139C | Sporulation-specific wall maturation protein                                                 | 18.4            | 18.2            | WT   |
| ECM33     | YBR078W | Involved in cell wall biogenesis and architecture                                           | 3.3             | 3.0             | 119  |
that was also observed to accumulate glycerol but less so than the wild-type strain (Fig. 2). Thus, the intracellular amount of glycerol was observed to decrease during PMKT treatment.

Hog1p Phosphorylation—HOG pathway signaling, which is manifested by the phosphorylation of Hog1p, was rapidly observed when cells were shifted to PMKT exposure. The phosphorylation of Hog1p was observed to occur in the first 10 min of exposure to PMKT, and only the first samples (0 and 5 min) failed to show a phospho-Hog1p signal. During the rest of the experiment the phospho-Hog1p signal was observed, pointing to permanent activation of the signaling pathway (Fig. 3).

YEAST DELETION MUTANTS AND SENSITIVITY TO PMKT—As may be seen in Table 1, there is a clear relationship between the level of induction of up-regulated genes and the presence of hypersensitive phenotypes in the mutants studied. In contrast, the particular case of hog1Δ mutant constructed for this study, found to be hypersensitive (180%) but with a very low up-regulation of its expression (0.4–0.8-fold expression), was very significant. Deletion mutants of genes known to be involved in a response to osmotic stress (YAP1, HAL9, GPP1, GPP2, GDP1, PDR12, PSR1, IST1, NHA1, ENA1, SAT2, TRK2, HSP12, CTT1, and CSF1) were highly hypersensitive to PMKT, and the glycerol synthesis-related mutants formed the group with the highest levels of hypersensitivity. On the other hand, there was no clear relationship between the deletion mutants obtained for down-regulated genes and the degree of toxin sensitivity (Table 2). In general terms, a marked tendency to obtain PMKT-resistant mutants (47%) and wild-type phenotypes (36%) was observed when repressed genes were deleted.

DISCUSSION

The response of the yeast S. cerevisiae to PMKT was studied by genomic expression profiling. Yeast genome scale DNA microarrays have been used to study global responses to chemical agents, antifungal agents, and environmental stresses (25, 29, 49–51). The gene expression response to PMKT was examined taking into account that the killing process of sensitive cells started 1 h after toxin addition in a process that supposedly involves several steps of the toxin mechanism of action. Because the binding of PMKT to cell wall receptors, (1–6)-ß-d-glucans, occurs in the first 2–3 min after toxin addition (6), the observed lag phase, which is necessary for changes in cell viability to be observed, probably involves several events (Fig. 4): adhesion to membrane receptors; formation of ion channels in the plasma membrane (10); metabolic and genetic changes, such as the dissipation of transmembrane electrochemical gradients (9) or the induction of stress response elements; and the formation of large membrane pores that allow the passage of large molecules either simultaneously to or sequentially with the formation of ion channels (10, 11, 52).

Correlation with Known Yeast Stress Responses—Comparison of the genes up-regulated in response to PMKT (Table 1) and those induced by osmotic stress (33) suggests that, with some exceptions, the genes induced in both cases would be similar. Therefore, the majority of PMKT-induced genes must encode proteins known or predicted to be involved in the osmotic response, thus indicating an underlying response to intracellular osmotic changes in response to the toxin. PMKT-induced genes include genes such as CTT1, HSP12, GDP1, GPP2, TRK2, PDR12, ENA1, YAP1, XBPI, SCH9, HAL9, and STL1 that are found to be directly related to cell stress or salt tolerance. Similarities were also found with the response of S. cerevisiae to polyene antibiotics such as amphotericin B and nystatin (51). Under environmental stress or treatment with certain polyene antibiotics it is very common to observe a reduction in the expression levels of some ribosomal proteins (29, 51, 53). This reduction is thought to be involved in the maintenance of a basal level of protein synthesis and in energy utilization to be redirected for increased expression of the genes involved in protective responses under these conditions. Our results indicated that mitochondrial ribosomal protein gene levels were not severely repressed and that nuclear ribosomal protein genes were not highly repressed (less than 2-fold repression). This difference in the genomic response to PMKT must be taken into account when the mechanism of killing is discussed because such differences between environmental stress and the action of polyene antibiotics could differentiate the underlying mechanisms of stress caused by different toxins, antibiotics, heavy metals, radiation, etc. These differences could plausibly be attributed to different observations over time of two similar processes, and hence another possibility is that there would be a different description of a succession of biochemical categories that are progressively up-regulated. This is justified because early stress responses usually affect mainly nucleotide and protein biosynthetic pathways and are different from later responses, which include intracellular protein and metabolite transport activities and increased energy consumption for metabolic and ion homeostasis. Transcription after prolonged stress also exemplifies ascending functions in cell rescue, aging (cell death), and defense-related roles.

Many induced genes during the response to PMKT were observed in the category of ion, sugar, amino acid, or multidrug transporters (Table 1). Among the ion transporters known to play a role in sodium detoxification, several transcripts were up-regulated (47, 48). It
# TABLE TWO

| Gene name | ORF     | Description of gene product | -Fold induction | PMKT death zone % |
|-----------|---------|------------------------------|-----------------|-------------------|
| **Signal transduction, gene expression, transcription, and RNA processing (11 genes)** | | | | |
| RGT1a     | YKL038W | Regulator of glucose-induced genes | 19.8 19.1 | WT |
| RDI1      | YDL135C | Rho GDP dissociation inhibitor with activity toward Rho1p | 3.1 3.0 | WT |
| YVIH1     | YIR026C | Protein tyrosine phosphatase | 4.2 3.8 | WT |
| MET18     | YIL128W | Involved in NER and RNA polymerase II transcription | 4.3 3.9 | 102 |
| HAP4      | YKL109W | CCAAT-binding factor subunit | 3.2 3.5 | 100 |
| MTF1      | YMR228W | RNA polymerase-specific factor, mitochondrial | 3.5 3.7 | 98 |
| PAB1      | YER165W | mRNA polyadenylate-binding protein | 4.3 3.9 | WT |
| NOP1      | YDL014W | Fibrillarin | 2.9 3.3 | WT |
| UTP4      | YDR324C | Nucleolar protein, component of the small subunit processome containing the U3 snoRNA | 2.0 2.4 | WT |
| RPF2      | YKR081C | Essential protein involved in the processing of pre-rRNA and the assembly of the 60 S ribosomal subunit | 3.8 3.3 | 105 |
| EFB1      | YAL003W | Translation elongation factor eEF1β | 3.0 3.5 | 95 |
| **Carbohydrate metabolism (six genes)** | | | | |
| RGT1a     | YKL038W | Regulator of glucose-induced genes | 19.8 19.1 | WT |
| ATF2      | YGR177C | Alcohol O-acetyltransferase | 3.9 3.2 | 90 |
| GPH1a     | YPR160W | Glycogen phosphorylase | 3.5 3.7 | 87 |
| TAL1      | YLR345C | Transaldolase isoform 1 | 3.3 3.5 | 92 |
| GND1      | YHR183W | 6-Phosphogluconate dehydrogenase | 2.9 3.1 | 96 |
| ACO1      | YLR304C | Aconitate hydratase (aconitase) | 3.2 3.2 | 103 |
| **Mitochondrial functions, energy and redox metabolism (five genes)** | | | | |
| GPH1a     | YPR160W | Glycogen phosphorylase | 3.5 3.7 | 87 |
| QCR9      | YGR183C | Ubiquinol cytochrome c reductase subunit 9 | 2.6 3.1 | WT |
| CYC1      | YJR048W | Cytochrome c isoform 1 | 3.2 3.4 | 93 |
| YTA12     | YMR089C | Protease of the SEC18/CDC48/PAS1 family of ATPases (AAA) | 3.0 3.0 | 95 |
| MBA1      | YBR185C | Respiratory chain assembly protein | 4.2 3.8 | 87 |
| **Amino acid and nucleotide metabolism (four genes)** | | | | |
| CHA1      | YCL064C | l-Serine/L-threonine deaminase | 9.2 10.2 | WT |
| FLR1      | YHR128W | Uracil phosphoribosyltransferase | 3.5 4.1 | 104 |
| MET6      | YER091C | 5-Methyltetrahydropteroyltriglutamate-homocysteine methyltransferase | 3.6 4.0 | 97 |
| SAM1      | YLR180W | S-Adenosylmethionine synthetase 1 | 5.6 5.8 | |
| **Lipid metabolism (four genes)** | | | | |
| ERG3      | YLR056W | C-5 sterol desaturase | 3.4 2.9 | WT |
| ERG25     | YGR060C | C-4 sterol methyl oxidase | 3.1 3.5 | WT |
| OLE1      | YGL055W | Stearoyl-CoA desaturase | 6.2 5.9 | 86 |
| FAA4      | YMR246 | Long chain fatty acid-CoA ligase | 3.6 3.4 | 89 |
| **Ionic homeostasis and transport facilitation (seven genes)** | | | | |
| FRE1      | YLR214W | Ferric and cupric reductase | 3.1 3.3 | 96 |
| BTN2      | YGR142W | Gene whose expression is elevated in a btn1 minus/ Btn1p lacking yeast strain | 4.5 4.6 | WT |
| PMP2      | YEL017C-A | H+ ATPase subunit, plasma membrane | 2.9 3.0 | 87 |
| PHO88     | YBR160W | Involved in phosphate transport, transmembrane protein | 3.1 3.2 | 96 |
| CTR1      | YPR124W | Cu2+ transport protein; required for high affinity uptake of Cu2+ | 3.3 4.0 | 104 |
| PMA1      | YGL008C | Plasma membrane proton ATPase | 2.5 2.6 | 87 |
| TPO2      | YGR138C | Member of the major facilitator superfamily multidrug resistance protein family | 3.8 3.4 | 92 |
| **Cell stress and rescue (five genes)** | | | | |
| HSP30     | YCR021C | Heat shock protein | 2.4 2.1 | 98 |
is known that the plasma membrane is the target of some killer toxins such as K1 (3, 11, 54), and the results of previous investigations exploring PMKT are consistent with the idea that the effect induced by the toxin lies in an alteration of the permeability of the yeast membrane (10). The leakage of micronutrients and ions induced by the formation of PMKT are consistent with the idea that the effect induced by the toxin lies in an alteration of the permeability of the yeast membrane (10). The leakage of micronutrients and ions induced by the formation of PMKT are consistent with the idea that the effect induced by the toxin lies in an alteration of the permeability of the yeast membrane (10). The leakage of micronutrients and ions induced by the formation of PMKT are consistent with the idea that the effect induced by the toxin lies in an alteration of the permeability of the yeast membrane (10). The leakage of micronutrients and ions induced by the formation of PMKT are consistent with the idea that the effect induced by the toxin lies in an alteration of the permeability of the yeast membrane (10). The leakage of micronutrients and ions induced by the formation of PMKT are consistent with the idea that the effect induced by the toxin lies in an alteration of the permeability of the yeast membrane (10). The leakage of micronutrients and ions induced by the formation of PMKT are consistent with the idea that the effect induced by the toxin lies in an alteration of the permeability of the yeast membrane (10). The leakage of micronutrients and ions induced by the formation of PMKT are consistent with the idea that the effect induced by the toxin lies in an alteration of the permeability of the yeast membrane (10). The leakage of micronutrients and ions induced by the formation of PMKT are consistent with the idea that the effect induced by the toxin lies in an alteration of the permeability of the yeast membrane (10). The leakage of micronutrients and ions induced by the formation of PMKT are consistent with the idea that the effect induced by the toxin lies in an alteration of the permeability of the yeast membrane (10). The leakage of micronutrients and ions induced by the formation of PMKT are consistent with the idea that the effect induced by the toxin lies in an alteration of the permeability of the yeast membrane (10). The leakage of micronutrients and ions induced by the formation of PMKT are consistent with the idea that the effect induced by the toxin lies in an alteration of the permeability of the yeast membrane (10). The leakage of micronutrients and ions induced by the formation of PMKT are consistent with the idea that the effect induced by the toxin lies in an alteration of the permeability of the yeast membrane (10). The leakage of micronutrients and ions induced by the formation of PMKT are consistent with the idea that the effect induced by the toxin lies in an alteration of the permeability of the yeast membrane (10). The leakage of micronutrients and ions induced by the formation of PMKT are consistent with the idea that the effect induced by the toxin lies in an alteration of the permeability of the yeast membrane (10). The leakage of micronutrients and ions induced by the formation of PMKT are consistent with the idea that the effect induced by the toxin lies in an alteration of the permeability of the yeast membrane (10).
represents a stress for organisms because the excess of sodium or other monovalent cations imbalances the osmotic potential and generates a water deficit, and the influx of sodium may lead to metabolic toxicity (57). ENA1, its transcriptional activator HAL9, and TRK2 were significantly induced after PMKT exposure, showing that PMKT generates an ionic or an osmotic stress in sensitive yeast cells. Additional genes involved in ionic stress and detoxification were up-regulated in the presence of PMKT, namely NHA1, IST1, TOK1, PSR1, PHO84, PHO89, PHO87, ZRT1, and CTR3.

Synthesis and Accumulation of Compatible Osmolytes—The behavior of many transcripts in our analyses correlated with known biochemical hyperosmotic stress responses. Glycerol, a compatible osmolyte known to accumulate rapidly in response to stress in yeast, accumulated transiently, and transcripts in the glycerol biosynthetic pathway increased (58). The transcripts of enzymes leading to glycerol production, GDP1 and GPP2, were increased during PMKT treatment (Fig. 1 and TABLE ONE). These observations agree with the fact that glycerol was observed to increase in response to PMKT (Fig. 2). Glycerol is produced in yeast by reduction of the glycolytic intermediate dihydroxyacetone phosphate to glycerol 3-phosphate followed by dephosphorylation. The first step is catalyzed by NAD-dependent glycerol-3-phosphate dehydrogenase, which is encoded by the two isogenes GDP1 and GPP2. GDP1 is induced under hyperosmotic stress, whereas the expression of GPP2 is relatively unaffected by hyperosmotic stress (59).

Mutants defective for GDP1 have an osmosensitive phenotype (27), and the double mutant gpd1Δ gpd2Δ is even more osmosensitive. The severity of these phenotypes is roughly proportional to the reduction in the cellular glycerol level (24, 60). Thus, the synthesis of glycerol is required for growth in a high osmolarity environment. According to Pagé et al. (61), Hog1 mutants show hypersensitivity to K1 killer toxin, but the wild-type phenotypes of gpd1Δ gpd2Δ double deletion mutants show that the mechanism of killing by K1 is not related to the accumulation of glycerol.

After the initial increment of the glycerol content, the intracellular amount of glycerol was observed to decrease in the presence of PMKT (Fig. 2). This observation is in accordance with the fact that the toxin enhances membrane permeability (10). When by different mechanisms a cell is de-energized, any ion or metabolite accumulated in the cell against its concentration gradient will tend toward equilibrium, and a flow will be observed (i.e. K+, ATP, and glycerol). This is consistent with the notion that the initial intracellular acidification of sensitive cells was observed simultaneously to the glycerol efflux 30–60 min after toxin addition and is in agreement with the fact that PMKT generates the outflow of ions such as potassium (Fig. 4). Another possibility would be that the presence of the killer toxin might generate channels through which glycerol could pass. This possibility, however, was not taken into account because of previous results obtained with patch clamp techniques suggesting that PMKT generates unregulated ionic channels (10).

Similarly to glycerol, trehalose metabolism genes, GLK1, PGM2, TSL1, TPS1, and NTH1, were up-regulated during PMKT activity. Trehalose is involved in yeast stress responses as an osmoprotectant, although it does not accumulate at osmotically significant concentrations in salt-stressed yeast (62, 63). PGM2, TPS1, and the regulatory factor encoded by TSL1 catalyze trehalose biosynthesis, whereas Nth1p (trehalase) leads to trehalose degradation and the formation of glucose (63). Completion of this cycle seems to be indicated by the up-regulated transcripts for the kinase GLK1. The presence of high transcript amounts for Nth1p and Glk1p may explain why the osmoprotectant trehalose does not accumulate during salt stress. Trehalose synthesis and degradation, in combination with glycerol production, play a key metabolic role in protection against high salinity; this notion, also based
on gene expression changes, has been proposed previously (62) and confirms the fact that PMKT generates a HOG response very similar to that obtained with a hyperosmotic stress.

To validate the data obtained from microarrays and Q-PCR that revealed the involvement of many HOG-regulated genes, Hog1p phosphorylation was examined in a *S. cerevisiae* sensitive strain (Fig. 3). Phosphorylation of Hog1p was rapidly observed when cells were shifted to PMKT exposure. The phospho-Hog1p signal was observed between 10 min after toxin addition and during the rest of the experiment, showing a permanent activation of the HOG signaling pathway. This result could be attributed to the permanent loss of glycerol (Fig. 2) from the sensitive cells due to the activity of the killer toxin and is confirmed by the observations made previously by different groups, who observed that mutants that are unable to produce or retain the osmolyte glycerol show strongly enhanced and sustained Hog1p phosphorylation (64). It has also been suggested that intracellular turgor pressure, caused by the glycerol level, leads to deactivation of the HOG pathway. This finding indicates that the kinetics of Hog1p phosphorylation and the subsequent induction and repression of genes are direct effects of PMKT. Taken together, the data also indicate the intricate interdependence of the PMKT mechanism of action, HOG signaling, and intracellular glycerol in the stress response of yeast sensitive to PMKT. Furthermore the observations from the Northern blotting experiments (Fig. 1) in which a permanent induction of mRNA synthesis was observed could reinforce the thought that the toxin generates a permanent stimulation of the cell because intracellular and extracellular ions are flowing through the plasma membrane simultaneously with some intracellular low molecular weight metabolites such as glycerol (Fig. 2). Finally the disruption of homeostasis across the plasma membrane could well lead to an increased mortality of the poisoned cells that, according to previously reported results, would not affect the physical integrity of the plasma membrane during the initial stages of the action of PMKT (10).

**General Stress Defense Response**—Whole-genome expression analysis has shown that the expression of more than 10% of *S. cerevisiae* genes is affected by several apparently unrelated stress conditions and that the exposure of yeasts to one type of stress strongly increases their capacity to resist another, different type of stress (25). The HOG signaling pathway of the yeast *S. cerevisiae* is defined by the *PBS2* and *HOG1* genes encoding members of the mitogen-activated protein kinase kinase and of the mitogen-activated protein kinase family, respectively. Mutations in this pathway (deletions of *PBS2* or *HOG1* or point mutations in *HOG1*) almost completely abolish the induction of transcription by osmotic stress that is mediated by stress response elements. These genes are induced by different stimuli such as oxidative stress, nutrient starvation, heat shock, and hyperosmotic shock. The percentage of up-regulated ORFs related to the defense response to PMKT was 16%. HSPs, which have been found to be up-regulated in a variety of stressful conditions such as oxidative stress, methyl methanesulfonate, and heat shock (53), were found to be up-regulated in response to PMKT (*HSP12, HSP104, HSP82, HSP26, DDR2, SSA2, SSB2, and SSA1*). The groups of HSPs induced during PMKT exposure were different from those induced in osmotic stress (65), indicating that different sets of HSPs could have different functional targets in the responses to osmotic stress or PMKT.

Our results also presented in TABLE ONE and Fig. 1 showed that *CTT1* (cytosolic catalase T) expression was increased with a PMKT treatment. Hog1-dependent osmotic induction of transcription of the *CTT1* gene occurs rapidly after an increase in osmolality (66). Consistent with a role of stress response elements in the induction of stress resistance, a number of other stress protein genes (e.g. *HSP104*) are regulated like *CTT1*. Furthermore catalase T has been shown to be important for viability under severe osmotic stress, and heat shock has been demonstrated to provide cross-protection against osmotic stress (67). Furthermore PMKT also induced *YAP1*, which specifically mediates oxidative stress responses and appears to be responsible for the rapid establishment of a transient transcriptional response. Yap1p, a relevant transcription factor that plays a major role in this response, binds, in vivo, promoters of genes that are not automatically up-regulated. It has been proposed that Yap1p nuclear localization and DNA binding are necessary, but not sufficient, to elicit the specificity of the chemical stress response (68).

**Yeast Deletion Mutants**—The *S. cerevisiae* gene deletion library offers a powerful tool for the assignement of new functions to sequenced genes. Because PMKT is an antifungal protein with unknown targets in yeast, it was of interest to identify genes contributing to PMKT sensitivity and resistance that might lead to further insight into the cellular mechanism of PMKT-mediated toxicity. We screened a library of single gene deletions for hypersensitivity to PMKT. The genes whose expression levels were seen to be regulated in response to PMKT were selected, and the deletion mutants for those genes were obtained. Deletion of up-regulated genes generatedhypersensitive phenotypes to PMKT. Several mutants of genes related with a osmotic stress response (*YAP1, HAL1, GPP1, GPD2, GPD1, PDR1, IST1, NHA1, ENA1, SAT2, TRK2, HSP12, CTT1*, and *CSF1*) were observed to be highly hypersensitive to PMKT, and the group of genes related to glycerol synthesis had the highest levels of hypersensitivity. With a general reading of these results we may assume that yeast cells respond to PMKT by increasing the expression of some genes that could counteract the effects of the toxin to some extent over a short period of time. Taking into account the fact that the mutations of down-regulated genes were observed to generate PMKT-resistant mutants or wild-type phenotypes, down-regulated genes could be involved in the mechanism of cell defense and rescue but in an indirect manner.

Regardless of certain possible additional effects, the results reported here indicate that PMKT generates a serious challenge for cell survival by disrupting plasma membrane electrochemical gradients, inducing a general stress response in a way comparable to that of some stimuli like hyperosmotic shock. The observed transcriptional response is consistent with the idea that the effect induced by PMKT lies in an alteration to the ionic homeostasis of the sensitive yeast cell. As such, the PMKT-induced stress response requires the simultaneous expression of several components of the sensitive yeast genome. In addition to producing a proper response to the stimulus of PMKT stress, the HOG pathway could play an important role in the response to PMKT. The mechanism of HOG-mediated response to PMKT is as yet unknown but will prove to be an exciting area of study in the future. Further studies are currently in progress to gain additional information about the properties of *P. membranifaciens* killer toxin in the hope of contributing to the understanding of a killer toxin with promising antifungal properties.

**Acknowledgments**—We are grateful to the Proteomic and Genomic Centre of Comunidad de Madrid and S. Menéndez from Central Radioactive Installation for expert technical assistant and to Dr. P. Vázquez and Dr. C. Sanz for helpful discussions.

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