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DNA microarray analysis suggests that zinc pyrithione causes iron starvation to the yeast *Saccharomyces cerevisiae*

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Zinc pyrithione has been used in anti-dandruff shampoos and in anti-fouling paint on ships. However, little is known of its mode of action. We characterized the effects of sub-lethal concentrations of zinc pyrithione (Zpt) on *Saccharomyces cerevisiae* using DNA microarrays. The majority of the strongly upregulated genes are related to iron transport, and many of the strongly downregulated genes are related to the biosynthesis of cytochrome (heme). These data suggest that Zpt induces severe iron starvation. To confirm the DNA microarray data, we supplemented cultures containing Zpt with iron, and the growth of the yeast was restored significantly. From these results, we propose that the principal toxicity of zinc pyrithione arises from iron starvation.

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[**Key words:** Zinc pyrithione; DNA microarray; *Saccharomyces cerevisiae*; Iron; Toxicity; Cytochrome]

1-Hydroxy-2-pyridinethione, or 2-mercaptopyridine N-oxide, also known as pyrithione (PT) under the trade name Omadine, is well known for its biocidal effect. PT is a lipophilic chelator, and its metal complex, zinc pyrithione (Zpt), has been used in anti-dandruff shampoos (1). It is also used as an anti-fouling paint, after The International Convention on the Control of Harmful Anti-fouling Systems on Ships banned organic tin (2, 3). Some studies have been carried out on the physiological and biochemical effects of PT. It interferes with thymidine uptake (4), and is suggested to be an anti-metabolite for vitamin B6 or nicotinic acid (5). Chandler and Segal (6) presented a hypothesis based on the finding that low concentrations of PT inhibited plasma membrane transport and promoted a decrease in cellular ATP levels and protein synthesis. PT was also reported to induce electrical depolarization of the membrane through inhibition of the proton pump (7). In that report, the effect of a range of Zn^{2+} concentrations (1 to 5 mM) on the membrane potential was also tested. However, no effect on the membrane potential was observed. Recently, PT has been used as a zinc ionophore to study zinc homeostasis in neurons in relation to Alzheimer's dementia (8). Furthermore, Zpt was found to inhibit cell growth strongly (9, 10). Dinning et al. (11) reported that both PT and Zpt reduced intracellular ATP levels in gram-negative bacteria, and that they are active against the cell membrane. However, its mode of action has not yet been elucidated clearly.

Saccharomyces cerevisiae is one of the most studied eukaryotic organisms, possessing a variety of advantageous characteristics as a model organism for bioassays. These include its relatively short life cycle, inexpensive media requirements and ease of handling. Recently, yeast microarrays have been used to monitor gene expression levels as a function of toxin exposure and as a means of determining the mechanisms of toxicity.

In this study, we exposed *S. cerevisiae* to sub-lethal concentrations of Zpt and analyzed the resultant genome-wide mRNA expression profiles. The results indicated that the yeast exhibited severe iron starvation and that Zpt appeared to act as a strong iron chelator or ionophore. This iron starvation caused heme depletion and may lead to decreased levels of ATP.

MATERIALS AND METHODS

Strains, growth conditions and chemicals The *S. cerevisiae* strain S288C (*Mat alpha SUC2 mal mel gal2 CUP1*) was grown as a pre-culture in YPD medium (2% polypeptone, 1% yeast extract, 2% glucose) at 25 °C for 2–3 days. This strain was chosen because the DNA microarray probes were produced using S288C as the template for the PCR reaction. The deletion strains, Δrad50, Δrad51 and Δrad52, which are derived from BY4743 (MATa/MATα his3Δ/*his3Δ leu2Δ0*/*leu2Δ0 ura3Δ0*/*ura3Δ0 met15Δ0*/*MET15 lys2Δ0*/*LYS2*), were purchased from Invitrogen (Carlsbad, CA, USA). Zinc pyrithione was purchased from Wako Pure Chemical Industries (Osaka, Japan).

It has been reported that Zpt is degraded rapidly by aqueous photolysis (12–14). Although normal laboratory light did not have a great influence on the result of the acute toxicity bioassay (15), we limited the exposure of the Zpt and Zpt-containing samples to light throughout these experiments as much as possible.

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The seed culture was diluted 1000-fold and grown overnight until an optical density ($OD_{660\ nm}$) of 1.0 was reached. In the dose-response experiments, Zpt was added to the cultures and the cultures were allowed to grow for a further 2 h. The cells were harvested by centrifugation and stored at −80 °C until use.

To observe the effect of iron supplementation on Zpt toxicity, we diluted the seed culture 1000-fold and allowed it to grow overnight in YPD at 30 °C, until an optical density ($OD_{660\ nm}$) of 1.0 was reached. Four serial 10-fold dilutions were made, and 8 µl of each of the five serially-diluted cell suspensions (including the culture) were spotted onto the indicated media and incubated at 30 °C for 3 days.

To determine the toxicity of Zpt to the Δ*RAD* strains, we serially diluted log phase cells ($OD_{660\ nm}$ = 1.0) of the deletion strains of Δ*RAD*s and their control strain (BY4743) that had been grown in YPD at 30 °C (the samples were diluted 10-fold at each step of the serial dilution). We spotted 8 µl of each of the five serial dilutions (including the undiluted culture) onto YPD agar plates containing the indicated concentrations of Zpt and incubated the plates at 30 °C for 2 days.

DNA microarray analysis Microarray experiments were carried out as described previously (16). Three independent cultures were used to isolate and purify poly $(A)^+$RNA. Messenger RNA from the Zpt-treated cells was fluorescently labeled with Cy5-dUTP, and the mRNA of the reference (untreated) cells was fluorescently labeled with Cy3-dUTP. Two color-labeled cDNAs were mixed and hybridized with a yeast microarray (ver. 2.0, DNA Chip Research, Inc., Yokohama, Japan) for 24–36 h at 65 °C. After hybridization, the labeled microarrays were scanned using a confocal laser ScanArray 4000 system (GSI Luminics, Billeria, MA, USA). The resulting image data were quantified using Quantarray (GSI Luminics). The signals that were detected for each open reading frame (ORF) were normalized using the intensity dependent (LOWESS) method. The genes that were classified as induced or repressed were those that passed a one-sample *t*-test (*P* value cutoff = 0.05) and that showed more than a two-fold increase or decrease in the expression, respectively, as compared to the control. The selected genes were characterized according to the categories of the Munich Information Center for Protein Sequences Genome Research Environment Comprehensive Yeast Genome Database (MIPS GenRE CYGD, http://mips.helmholtz-muenchen.de/genre/proj/yeast/index.jsp) and the Saccharomyces Genome Database (SGD, http://www.yeastgenome.org/). The data obtained from this experiment are available using the accession number GSE9223 in the Gene Expression Omnibus Database (GEO, http://www.ncbi.nlm.nih.gov/geo/).

RESULTS

The effects of Zpt on the growth of yeast The growth of *S. cerevisiae* S288C was inhibited at a concentration of 0.32 µM Zpt and slowed drastically at 1.57 µM Zpt (Fig. 1). A variety of Zpt concentrations were added to 50-ml cultures ($OD_{660\ nm}$ = 1.0) with shaking, and the number of surviving cells was counted. In the absence of Zpt, the numbers of yeast doubled (cfu/ml), but in the presence of 1.26 µM Zpt, the number of viable cells did not change during 2 h of treatment (data not shown). Generally, we did not detect a significant change in the yeast transcriptional profile when we used Zpt concentrations that were sufficiently low as not to affect growth, and we were unable to prepare sufficient mRNA from the cells that had been treated with concentrations so high that the cells died (17). Therefore, we extracted mRNA from yeast that had been treated with 1.26 µM Zpt.

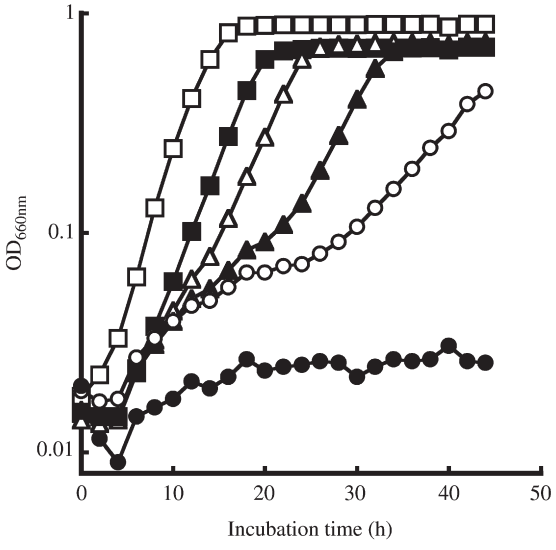

FIG. 1. The effect of Zpt on the growth of *S. cerevisiae* S288C. *S. cerevisiae* S288C were grown in YPD supplemented with various concentrations of Zpt at 25 °C: the concentrations were 0 µM (open square), 0.32 µM (solid square), 0.63 µM (open triangle), 1.26 µM (solid triangle), 1.57 µM (open circle), and 3.15 µM (solid circle), respectively.

Treatment with Zpt resulted in iron starvation of the yeast cells After obtaining three sets of microarray data (GSE9223), we used the average ratios (Cy5 of the Zpt-treated cells/Cy3 of the control) of the hybridization intensity for analysis. Ratios of the hybridization intensity (Cy5/Cy3) higher than 2.0 were considered upregulated and ratios less than 0.5 were considered downregulated. 157 ORFs (2.5%) showed a ratio of more than 2.0 and 239 ORFs (3.9%) showed a value of less than 0.5.

Seven out of the eight genes (*FIT1*, *FIT2*, *ARN2*, *FIT3*, *ARN1*, *FRE2* and *HMX1*) that had been upregulated more than 10-fold were related to iron metabolism (Table 1) (the other gene that was upregulated was *FRM2*). In addition, one half of the ORFs in the top 20 downregulated genes were related to the respiratory electron transport chain (*SDH3, CYT1, RIP1, CCP1, CYC1, QCR7, HEM15, COX6* and *COX4*). The list of genes that are highly induced suggests that the yeast is struggling to import iron ions, and the list of repressed genes suggests attempts to conserve iron ions. These most upregulated and downregulated genes strongly indicate iron starvation in the yeast cells. Other iron transporter genes, *SIT1* (6.54-fold), *FTR1* (4.57-fold), *FET3* (4.24-fold) and *FRE3* (4.19-fold) were induced more than four-fold. In addition, the iron metabolism genes *FET5*, *ENB1* and *FRE6* were induced more than two-fold. Based on the upregulation of the iron transport genes, we tested the effect of iron supplementation on Zpt treatment. As shown in Fig. 2, supplementation of ferrous sulfate (2 mM) relieved the growth inhibition resulting from the treatment with 1.26 µM Zpt (due to presumed iron starvation) almost completely. The inhibition caused by 2.52 µM Zpt was partly alleviated.

Zpt also led to a metal ion metabolism disorder Together with iron transporter genes, some other metal transporting ORFs were also induced following exposure to Zpt treatment. These included *CCC2* (a copper transporting P-type ATPase, which has been reported to be indispensable in iron transport (18, 19), 2.98-fold), *COT1* (a vacuolar zinc transporter, 2.77-fold), *ATX1* (a cytosolic copper metallochaperone, 2.56-fold), *PCA1* (a P-type ATPase that plays a role in copper and iron homeostasis and controls the intracellular cadmium level, 2.52-fold), *ATX2* (a Golgi membrane protein that plays a role in manganese homeostasis, 2.33-fold) and *ZRT3* (vacuolar zinc efflux protein, 2.10-fold). Two genes that have been reported to be induced by low levels of copper ions, *FRE7* (20) and *CTR1* (21), were the most repressed ORFs following Zpt treatment (Table 1). However, the copper metallothionein genes, *CUP1-1*, *CUP1-2*, and *CRS5*, which were the genes that were induced the most by treatment with copper (22), were not induced more than two-fold. A possible reason for the severe downregulation of *FRE7* and *CTR1* may be that this occurs in an attempt to compensate for indiscriminate metal ion intake, rather than as a response to copper toxicity that arises as a side effect of Zpt. These data confirm that yeast that had been treated with Zpt enhanced their metal ion incorporation activity.

Table 2 lists the genes that contribute to zinc metabolism. The *COT1* gene, whose product is thought to pump zinc from the cytosol to the vacuole, was induced 2.77-fold. However, the *ZRC1* gene, which also encodes a vacuolar transporter that responds to the accumulation of zinc in the cytosol, was not induced (0.73-fold). The transcription factor, Zap1p, increased the expression of some target genes in response to zinc deficiency (23). Two target genes, *ZRT1* and *ZRT2*, encode plasma membrane zinc transporters which were repressed 0.63- and 0.29-fold, respectively. However, other target genes such as

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