RESEARCH ARTICLE

Exposure to the lampricide TFM elicits an environmental stress response in yeast

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One sentence summary: Yeast cultures exposed to lampricide, a pesticide used to control the sea lamprey population in lakes, alters expression of many yeast stress response genes.

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ABSTRACT

The pesticide 3-trifluoromethyl-4-nitrophenol (TFM) is used to control sea lamprey populations in the Great Lakes and Lake Champlain. Little is known about the effects of this pesticide on gene expression in eukaryotic species. This study used microarray analysis to determine the effects of short term, high dose TFM exposure on gene expression in *Saccharomyces cerevisiae*. Yeast grown in standard glucose-containing media showed significant variation in gene expression in pathways related to cytoplasmic translation with the majority of these genes being downregulated. These findings were supported by the analysis of a similar but glucose-free experiment revealing that these cytoplasmic translation genes, mostly ribosomal subunit expressing genes, were similarly downregulated. The repression of the ribosomal subunit genes suggests that TFM exposure, regardless of glucose availability, evokes features of the environmental stress response in yeast.

Keywords: TFM; gene expression; *Saccharomyces cerevisiae*; cytoplasmic translation; environmental stress response

INTRODUCTION

Baker's yeast, or *Saccharomyces cerevisiae*, has long been used as a eukaryotic model organism to investigate the cellular and molecular effects of environmental stressors (Sánchez and Nielsen 2015). The extensive base of literature and genomic information on yeast coupled with the simplicity of working with this organism make it an ideal model system to elucidate the effects of chemicals and other environmental challenges on eukaryotic cells (Taymaz-Nikerel, Cankorur-Cetinkaya and Kirda 2016). Several pivotal studies have shown that yeast respond to a variety of stressors (e.g. hydrogen peroxide exposure, heat shock, amino acid starvation, etc.) with strikingly similar changes in gene expression (Gasch et al. 2000; Cauton et al. 2001; reviewed in Taymaz-Nikerel, Cankorur-Cetinkaya and Kirda 2016). Subsequently named the 'environmental stress response' (ESR), this phenomenon suggested that yeast utilize

similar cellular pathways to respond to a multitude of environmental challenges. According to Gasch et al. (2000), of the approximately 900 genes that intersected between the different conditions, around 600 genes showed patterns of repression; these included genes involved in cytoplasmic translation processes such as RNA metabolism and ribosome biosynthesis. Approximately 300 genes showed patterns of induction and included genes related to stress defense responses including cell wall alteration, DNA repair mechanisms, and carbohydrate metabolism.

Microarray analysis in yeast has been utilized to analyze the effects of several chemicals used to control invasive organisms, including the fungicides thiuram, zineb, maneb, chlorothalonil (TPN), and pentachlorophenol (Kitagawa et al. 2002; Kitagawa, Momose and Iwahashi 2003) and the herbicides glyphosate, sulfometuron methyl and 2,4-dichlorophenoxyacetic acid

(Jia et al. 2000; Sirisattha et al. 2004; Teixeira et al. 2006). While each treatment resulted in alterations in cellular pathways specific to each chemical, the majority of these treatments also resulted in induction and repression of genes commonly altered in the ESR. For example, treatment with the herbicide 2,4-dichlorophenoxyacetic acid, a phenol-containing pesticide, revealed alterations in pathways common to the ESR including a downregulation of genes related to transcription and translation and an upregulation of genes involved in the oxidative stress response (Teixeira et al. 2006).

The goal of this study was to test whether exposure to 3-trifluoromethyl-4-nitrophenol (TFM), a pesticide used in Lake Champlain and the Great Lakes to control sea lamprey populations, evokes the ESR gene expression changes in yeast. TFM is a hydrophobic, phenolic weak acid that releases hydrogen ions in the mitochondrial matrix, disrupting the hydrogen ion gradient required to fuel ATP synthesis (Drysdales and Cohn 1958; Birceanu et al. 2011). To determine global gene expression alterations due to TFM exposure, yeast grown in standard, glucose-containing (unrestricted) media were treated with 0.05 mM TFM over a four hour period; microarray analysis revealed that genes involved in cytoplasmic translation were significantly downregulated. To validate the results, a comparison was made with a dataset from another experiment where yeast grown in glucose-free (restricted) media was exposed to TFM. The results suggest that, regardless of growth media, exposure to TFM evokes the repression of cytoplasmic translation genes, a well-characterized feature of the ESR in yeast.

MATERIALS AND METHODS

Yeast strains, growth conditions and TFM treatment

An independent microarray dataset was generated from an experiment in which yeast cultures grown in glucose-containing (unrestricted) medium were treated over time with TFM. The wild type *S. cerevisiae* strain BY4741 (MAT α his3 Δ 0 leu2 Δ 0 met15 Δ 0 ura3 Δ 0) was used. Cultures were grown in yeast peptone dextrose (YPD), a fermentable medium (1% yeast extract, 2% peptone, and 2% dextrose), at 30°C to saturation and treated for 1, 2 or 4 h with 0.05 mM TFM in 2% isopropanol (TFM solvent). TFM stock solutions (5 mM) were made with isopropanol as the solvent and 0.5 ml stock solution was added to 50 ml broth cultures of yeast to final working concentration of 0.05 mM. The TFM powder was 99% pure ordered from Sigma Aldrich. The TFM dose of 0.05 mM (~10 mg/L) was chosen since it was the highest tested that showed no growth inhibition when cultures of yeast were exposed long term; typical field concentrations are 1.3–1.5X the mean lethal concentration (~2 mg/L). Cell viability was measured by agar plating and optical density measurement as in Hinkle et al. (2016) and there was no change in viability in the TFM treated versus control treatment.

RNA isolation, cDNA and cRNA synthesis and microarray analysis

For each TFM treated time point, two independent RNA samples (two biological replicates) were isolated (except for at time 4 h, where only one RNA sample was isolated but two technical replicates were prepared and processed). The two biological replicates taken immediately before treatment represented 'time zero', untreated (2% isopropanol only) controls. As described in Tighe et al. (2011), yeast samples were washed and enzymatically lysed with lyticase (Sigma) and total RNA was iso-

lated using RNeasy Mini Kits (Qiagen). RNeasy columns were pre-treated with DNase I (Qiagen) to remove contaminating genomic DNA. RNA quality and quantity were assessed by agarose gel electrophoresis and by NanoDrop spectrophotometry.

One μ g of total RNA was used in the SuperScript II reverse transcriptase assay (Invitrogen) using the T7 oligo-dT₂₄ primer and second strand cDNA synthesis kit per manufacturer's instructions. Phenol-chloroform-isoamyl alcohol extraction was performed to clean up the cDNA followed by in vitro transcription using an Enzo bioarray kit. RNeasy spin columns (Qiagen) were utilized to clean up cRNA, which was then fragmented using the hammerhead cleavage reaction (Tighe et al. 2011). Fragmentation was validated by agarose gel electrophoresis. Hybridization of cRNA to Yeast 2.0 GeneChips (Affymetrix) and streptavidin-phycoerytherin and biotinylated anti-phycoerytherin antibody staining was performed at the University of Vermont in the Vermont Genetics Network Microarray Facility. Chips were scanned in a high resolution GS3000 scanner, and data was processed.

Data preprocessing and statistical analysis

The experiment as described in the preceding section where cells were grown in glucose-containing YPD media will be referred to as the unrestricted experiment. To validate the results from the unrestricted experiment, a comparison was made to a similar microarray experiment (NCBI Gene Expression Omnibus database accession number GSE74259) that utilized the same yeast strain, treatment durations and TFM dose, but yeast cultures were grown in a restricted medium (forced aerobic metabolism), yeast peptone glycerol ethanol (Hinkle et al. 2016). This experiment will be referred to as the restricted experiment. The unrestricted experiment included untreated samples taken only at time zero and TFM treated samples at each subsequent time point (1, 2 and 4 h), whereas the restricted experiment included both TFM treated and untreated samples for comparison at each time point (1, 2 and 4 h).

The method for analyzing the data from the unrestricted and restricted experiments was the same. Both datasets were pre-processed separately using the robust multi-array analysis normalization method (Wu et al. 2004; Gillespie et al. 2010) where the raw intensity values were background corrected, log base 2 transformed and normalized using quantile normalization. The normalized values were fitted with an additive model to obtain an expression measure for each probe on the chip. The Affy-PLM R package (Bolstad et al. 2005) available from Bioconductor (www.bioconductor.org) was used to perform the quality control assessment and preprocessing of the data on each dataset separately.

The statistical analysis method Linear Models for Microarray data (*limma*) (Smyth 2004) was used to analyze the data. The datasets were analyzed separately using the *limma* package (Ritchie et al. 2015) from Bioconductor. For the unrestricted experiment, the reference for differential expression at each time point is in comparison to the expression values at time zero (0 h), the control in this experiment. For the restricted experiment, the reference for differential expression is between the TFM treated samples and untreated samples. The gene ontology (GO) enrichment tool from the *Saccharomyces* Genome Database (SGD; www.yeastgenome.org) was used to group genes based on biological process.

To control for the false discovery rate, the Benjamini-Hochberg procedure was used to adjust the *P*-values in the *limma* and the GO enrichment analysis. An adjusted *P*-value less than

Figure 1. Counts of differentially expressed genes at each time point in TFM treated yeast versus the control at time zero in the unrestricted experiment. The Venn diagram displays the counts of differentially expressed genes between the three comparisons (1 vs 0 h, 2 vs 0 h, and 4 vs 0 h); using the Benjamini-Hochberg correction procedure for the false discovery rate, an adjusted *P*-value less than 0.05, and an absolute value of a log fold change of at least 1/2 to determine differential expression.

0.05 and an absolute value of log fold change of 1/2 or more was used to determine differential expression for each of the genes.

RESULTS

Analysis of microarray dataset from TFM treated yeast in glucose media

To test whether exposing yeast cells to TFM evokes gene expression changes reflecting the environmental stress response, yeast cultures grown in unrestricted glucose media were exposed to TFM for 1, 2 or 4 h and microarray analysis was conducted. Using *limma*, 730 unique genes were identified as differentially expressed at one or more time points in comparison to the expression at time zero, the control for this experiment. The Venn diagram (Fig. 1) displays the counts of differentially expressed

genes between the three comparisons (1 vs 0 h, 2 vs 0 h and 4 vs 0 h).

The 730 genes were analyzed using the SGD to understand which biological processes were affected by TFM exposure. Of the 730 genes, 726 were annotated on the SGD. The GO enrichment tool from SGD revealed 77 significant GO terms for biological processes (Supplemental Data, Table S1). The top 10 GO terms are given in Table 1. Ribonucleoprotein complex biogenesis (125 genes) is a parent term for all of the GO terms except for cytoplasmic translation (24 additional genes), ncRNA processing (10 additional genes) and rRNA metabolic process (3 additional genes, 1 in ncRNA processing). Thus, there were a total of 161 unique genes in the top 10 GO terms.

To determine whether the 161 genes were part of the ESR, a comparison was made between these genes and those from other studies that involved exposing yeast to environmental stressors. Initially, the list was compared against approximately 900 genes listed by Gasch et al. (2000) as environmental stress response genes; 124 of the 161 genes were found to be among this group. The remaining 37 genes were identified as ESR genes in subsequent studies as found by using the yStreX database (<http://www.ystrexdb.com/>). This online database was created by Wanichthanarak, Nookaew and Petranovic (2014) as a repository of data on gene expression from 82 microarray datasets from a total of 121 environmental conditions. All of the genes in the top 10 GO terms were found in other studies to be affected by some environmental change.

Validation of gene expression changes using dataset from glucose-free, TFM treated yeast

The data from a previously published dataset in which yeast were treated with TFM in a restricted (glucose-free) medium (Hinkle et al. 2016) were used to validate the findings in the unrestricted (glucose-containing) experiment. Of the 161 genes that were differentially expressed in the unrestricted TFM exposure experiment, 124 of these genes were considered differentially expressed in the restricted experiment (Supplemental Data, Table S2). A comparison of the patterns of gene expression profiles over time between these two datasets revealed a common heat map profile for genes related to cytoplasmic translation (Fig. 2). Restricting the focus of examination to the 57 genes listed in the cytoplasmic translation GO term showed that 54 of the 57 genes had similar expression profiles in both the unrestricted and the restricted experiment (Fig. 2), with both conditions showing a downregulation at 4 h of TFM exposure. The

Table 1. The top 10 significant GO terms for biological processes shared by the differentially expressed genes in the unrestricted experiment using the GO Enrichment tool from the SGD.

	GO term	Accession number	P-value	Matches
1	ribosome biogenesis	GO:0042254	2.87E-18	119
2	ribonucleoprotein complex biogenesis	GO:0022613	3.43E-14	125
3	rRNA metabolic process	GO:0016072	3.94E-13	91
4	rRNA processing	GO:0006364	3.99E-13	88
5	ribosomal large subunit biogenesis	GO:0042273	1.62E-12	45
6	ribosomal small subunit biogenesis	GO:0042274	1.63E-11	49
7	cytoplasmic translation	GO:0002181	3.31E-11	57*
8	maturation of SSU-rRNA	GO:0030490	7.29E-10	41
9	ncRNA processing	GO:0034470	1.13E-09	98
10	maturation of SSU-rRNA from tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA)	GO:0000462	1.43E-09	38

*The GO Enrichment tool gave 56 as the count of matches for cytoplasmic translation but listed 57 genes total. The analysis is done on the 57 genes listed for the cytoplasmic translation from the SGD.

Figure 2. Comparative heat maps of the expression profiles in both the TFM exposed, unrestricted and restricted experiments. Shown is a comparison of the 57 genes identified at differentially expressed in the unrestricted experiment and listed in the GO term cytoplasmic translation. Red indicates upregulation and green indicates downregulation. Three genes that were identified as differentially expressed in the unrestricted dataset were not found to be differentially expressed in the restricted dataset (marked with *). The last three genes in the heat maps did not have a similar expression profile in the two experiments; in the unrestricted experiment there is upregulation of expression at one hour, whereas, in the restricted experiment gene expression is downregulated at four hours.

majority of these genes (48) code for large or small ribosomal subunit proteins (Supplemental Data, Table S2). Representative gene expression profiles of two of these genes (one large ribosomal subunit, one small ribosomal subunit) are shown in Fig. 3.

DISCUSSION

The routine use of TFM as a lampricide in the Lake Champlain and Great Lakes basins was the impetus to examine the eukaryotic cellular and molecular consequences from exposure to this chemical. *Saccharomyces cerevisiae* is often utilized to model gene expression changes to stressors and other environmental changes in eukaryotic systems. As such, cultures of yeast were exposed to TFM to determine temporal cellular alterations that occur in the presence of this pesticide. The major feature of the ESR that was detected in the TFM exposure experiment was the significant downregulation of expression for genes related to cytoplasmic translation. It is known that ribosome subunit biosynthesis (large or small) and cytoplasmic translation utilize significant cellular resources in normal growth conditions in the absence of stressors (Warner 1999; Ho and Gasch 2015). Despite glucose availability and the possibility to switch completely to anaerobic fermentation in the presence of TFM, cytoplasmic translation pathways were downregulated, suggesting an acute stress response. This was validated using the data from the restricted experiment, suggesting that, independent of glucose availability, TFM exposure downregulates the capacity of the cell to perform protein translation by downregulating ribosome subunit synthesis. Downregulation of genes involved in

cytoplasmic translation pathways and in protein synthesis may lead to growth inhibition with longer TFM exposure.

Some biological pathways common in the ESR were not differentially expressed in the unrestricted or restricted experiments. For example, genes that are responsive to Msn2 and Msn4 transcription factors (Martínez-Pastor et al. 1996; Taymaz-Nikerel, Cankorur-Cetinkaya and Kirda 2016) that are commonly upregulated in the ESR were not identified as differentially expressed in either experiment. Msn2/4 bind to the stress response element to regulate gene expression during nutrient depletion (reviewed in Morano, Grant and Moye-Rowley 2012); hence, it was hypothesized that genes regulated by Msn2/4 might change in this experiment. One possible explanation that there were no changes in Msn2/4 regulated gene expression is that both TFM exposure studies (restricted and unrestricted) focused on acute, early response differences in gene expression (0–4 h TFM exposure). In fact, in cold shock experiments in which the environmental stressors are temperature shifts (Schade et al. 2004), Msn2/4 responsive genes were altered only in long term cold exposure (≥ 12 h), suggesting that induction of these genes is related to the adaptation to stress rather than to the acute changes in biological pathways as an immediate response to stressors. Longer duration TFM exposures would reveal whether Msn2 and Msn4 responsive genes are altered with this environmental challenge.

Since TFM is a phenolic pesticide known to disrupt aerobic respiration, it is interesting to compare and contrast pathways altered in microarray experiments in which yeast were exposed to other chemically similar pesticides. Indeed, exposure to the herbicide 2,4-dichlorophenoxyacetic acid (Teixiera et al. 2006)

Figure 3. Expression profiles over time for two representative genes from the GO term cytoplasmic translation (57 genes total) found to be differentially expressed in both the unrestricted and restricted experiments. All expression levels are log base 2. The dark green line with the symbol 'O' represents the expression profile for the TFM treated yeast in the unrestricted experiment. The reference for differential expression in the unrestricted experiment is the expression level at time zero, the control for this experiment (two biological replicates at 0, 1 and 4 h, and two technical replicates at 2 h). The solid line with the symbol 'T' represents the expression profile of each gene for TFM treated yeast in the restricted experiment (two biological replicates at 1, 2 and 4 h). The solid line with the symbol 'U' represents the expression profile of each gene for untreated yeast in the restricted experiment (two biological replicates at 0, 1 and 4 h, and only one replicate at 2 h). The reference for differential expression in the restricted experiment is between the treated samples and untreated samples.

resulted in the upregulation of genes related to cell wall assembly similarly found in the TFM exposure in the unrestricted experiment. These pesticide exposure studies also revealed a downregulation of the target of rapamycin (TOR) signaling family of genes which act to promote protein translation and ribosome biogenesis (Powers and Walter 1999; Lushchak et al. 2017). While ribosome subunit biogenesis was downregulated in the TFM exposure experiments, only approximately 30% of genes in the TOR pathway were differentially expressed in the unrestricted experiment. The fact that TFM exposure alters expression in ribosome subunit biogenesis but not TOR and/or Msn2/4 pathways suggests unique cellular responses to TFM in comparison to other pesticides.

Comparing the results from the unrestricted and restricted experiments gives insight into the variations in gene expression due to TFM exposure that were related to glucose availability. As expected, the restricted experiment resulted in a much higher number of differentially expressed genes (730 in the unrestricted versus 2468 in restricted). Yeast in the glucose-deficient, restricted medium were more metabolically challenged by the TFM exposure than those in the unrestricted medium because of the lack of glucose, which limited their ability to utilize anaerobic glycolysis to meet the ATP demands of the cells when exposed to TFM. While many other biological processes characteristic of the ESR were significantly altered in the unrestricted dataset (e.g. cell wall bioassembly), these processes were not validated in the restricted experiment, suggesting that these

changes may be related to glucose availability. As reported in Hinkle et al. (2016), the most significant GO term identified for the restricted dataset was 'carbohydrate metabolism' with hexose transporters being significantly upregulated. However, this was not seen in the unrestricted experiment suggesting that although TFM exposure may make aerobic respiration less efficient, the availability of glucose for uptake by the hexose transporters would allow for anaerobic glycolysis to continue. In contrast, the lack of glucose coupled with TFM exposure in the restricted experiment likely led to the increase of expression of hexose transporters seen at 4 h; this might be via a feedback mechanism where the cell responds to the restricted, glucose starved condition by upregulating these transporters.

The continued use of TFM in Lake Champlain and Great Lakes watersheds to control sea lamprey populations warrants a better understanding of cellular and molecular alterations resulting from exposure to the pesticide. The evocation of some components of the ESR in yeast with TFM exposure suggests that use of this chemical may result in similar cellular changes in target and non-target species. Interestingly, a recent TFM-exposure study in rainbow trout revealed an elevation of cortisol levels that persisted for 12 h post treatment, suggesting a metabolically protective physiologic response to TFM exposure in fish (Birceanu and Wilkie 2018). The molecular study here revealed potential short-term biomarker genes in eukaryotes whose altered expression patterns may also inform the understanding of non-target species effects. Future gene expression studies

utilizing higher organisms, including species directly impacted by lampricide exposure, will determine which environmental stress conditions likely arise in exposed aquatic organisms.

DATA AVAILABILITY

Microarray data is available in the National Center for Biotechnology Information Gene Expression Omnibus database under accession number GSE101178.

SUPPLEMENTARY DATA

Supplementary data are available at [FEMSyr](https://www.femsy.com) online.

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