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Exposure to the lampricide 3-trifluoromethyl-4-nitrophenol results in increased expression of carbohydrate transporters in *S. cerevisiae*

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Abstract

The lampricide 3-trifluoromethyl-4-nitrophenol (TFM) is used to control sea lamprey (*Petromyzon marinus*) populations in freshwater lakes. While TFM can have sublethal and lethal effects, little is known about gene expression changes with TFM exposure. Microarray analysis was used to determine differential gene expression over 4 hours of exposure in *S. cerevisiae*. Among the most significantly up regulated genes were regulators of carbohydrate transport including HXT1, HXT3, HXT4, IMA5, MIG2, and YKR075C.

Keywords

TFM; Gene expression; *Saccharomyces cerevisiae*; Carbohydrate transport; Microarray

INTRODUCTION

The chemical lampricide 3-trifluoromethyl-4-nitrophenol (TFM) has been used for decades in the Great Lakes and Lake Champlain basins to reduce the population of sea lamprey (*Petromyzon marinus*), predatory fish that have contributed to the decline of trout, salmon, and other game fish [1,2]. Typical TFM protocols in the field include constant application of the chemical over 12–14h at tributary headwaters approximately every 4 years with the intent to kill sea lamprey larvae [1]. TFM doses range widely (typically 1–8 mg/L) as the minimum lethal concentration to kill 99.9% of the target species is dependent on pH, alkalinity, flow rate, water temperature, and other variables. While stream ecosystems recover rapidly after TFM treatment, non-target species mortality has been reported in algae [3], aquatic insects [4,5], fish [6,7], and amphibians [8,9]. Controlled laboratory toxicology studies showed mild to severe toxicity in juvenile lake sturgeon, tadpole madtom, and

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SUPPLEMENTAL DATA

Tables S1-S2 and Figure S1.

Data availability

Microarray data have been submitted to NCBI GEO database under accession number GSE74259.

channel catfish [10]. These studies indicate that TFM treatment may affect non-target species and underlie the importance of elucidating the cellular and molecular mechanisms resulting from TFM exposure.

Similar to the classic uncoupling chemical 2,4-dinitrophenol (DNP), 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 [11,12]. Studies in rat mitochondria [13], trout [14], and sea lamprey larvae [15,16,17] have shown that TFM uncouples oxidative phosphorylation from ATP synthesis, leading to ATP deficits, a reduction in glycogen reserves, and increased oxygen consumption. Interestingly, it has also been shown that TFM is an estrogen receptor agonist [18] and that it alters testosterone levels in fish [19] suggesting that TFM may cause transcriptional changes in cells and may act as an endocrine disruptor as well as a metabolic uncoupler.

Despite the relative success of TFM treatment protocols in reducing sea lamprey populations, little is known about alterations that occur on the molecular level as a result of exposure to this pesticide. The overall goal of the present study was to use global gene expression analysis in *S. cerevisiae* to determine altered biological processes in eukaryotic cells that result from TFM exposure. The extensive base of literature and genomic information available for yeast make it an ideal model system to elucidate the effects of chemicals and other environmental challenges on eukaryotic cells; moreover, the conservation of basic cellular functions between yeast and other eukaryotic species makes it an ideal and often utilized system by which to test toxicologic effects [20]. After analyzing growth characteristics and viability in *S. cerevisiae* with varying TFM concentrations, a time course microarray study was performed that revealed significantly altered patterns of gene expression when yeast cultures were exposed to 0.05mM TFM over 4 hours. The present study represents the first global gene-specific analysis of differential gene expression due to TFM treatment.

METHODS

TFM doses and *S. cerevisiae* growth and viability studies

TFM doses were based on recent controlled studies in sea lamprey larvae that tested toxicity with TFM concentrations ranging from 0.01–0.120mM [10,17]. *S. cerevisiae* strain BY4741 (*MATa his3 0 leu2 0 met15 0 ura3 0*) was used in all experiments in the present study. Cultures were grown in yeast peptone dextrose (YPD), a fermentable media (1% yeast extract, 2% peptone, and 2% dextrose), or yeast peptone glycerol ethanol (YPGE), a non-fermentable media (1% yeast extract, 2% peptone, 2% glycerol, and 2% ethanol), at 30°C with vigorous shaking at 250rpm. For growth curve analysis, early-log phase yeast cultures with an optical density at 600nm (OD₆₀₀) of approximately 0.5 were treated with 0.05mM, 0.1mM, or 0.5mM TFM (Sigma) or 2% isopropanol (solvent control) and OD₆₀₀ measurements were recorded at multiple time points over 24–28h. Three independent trials were performed and averaged to generate growth curves. For viability studies, early-log phase cultures were treated for 1h, 4h, 10h, or 24h with 0.05mM, 0.1mM, or 0.5mM TFM or with equal volumes of 2% isopropanol or water. Cells were diluted (10^{-5}) and plated onto

YPGE or YPD agar, plates were incubated for 24h at 30°C, and colonies were counted. Viability results represent averages from 3 independent trials.

RNA isolation and microarray analysis

Cultures of BY4741 yeast were grown in non-fermentable YPGE media at 30°C to saturation and treated for 1h, 2h, or 4h with 2% isopropanol or 0.05mM TFM. Samples taken immediately before treatment represented “time zero” samples. For each treatment and time point, 2 independent RNA samples were isolated (except at 2h for untreated control yeast due to poor RNA quality of 1 of the samples). Preparation of RNA samples was performed as in Tighe et al. [21]. Total RNA was isolated from enzymatically-lysed *S. cerevisiae* using RNeasy Mini Kits (Qiagen) per manufacturer’s instructions. RNeasy columns were pre-treated with DNase I (Qiagen). RNA was quantified by spectrophotometry (OD₂₆₀). Quality and purity were assessed by agarose gel electrophoresis and by taking OD_{260:280} and OD_{260:230} ratios.

First and second strand cDNA synthesis and in vitro transcription were performed as in Tighe et al. [21] using the SuperScript II reverse transcriptase assay with T7 oligo-dT₂₄ primer and second strand cDNA synthesis kit per manufacturer’s instructions (Invitrogen). cDNA cleanup was performed by phenol-chloroform-isoamyl alcohol extraction, and in vitro transcription was performed using an Enzo bioarray kit. cRNA cleanup was performed using RNeasy spin columns (Qiagen). cRNA was fragmented using the hammerhead cleavage reaction [21] and fragmentation was assessed by agarose gel electrophoresis.

Hybridization of cRNA to Yeast 2.0 GeneChips (Affymetrix) and subsequent streptavidin-phycoerytherin and biotinylated anti-phycoerytherin antibody staining was performed at the Vermont Genetics Network Microarray Facility at the University of Vermont. Chips were scanned in a high resolution GS3000 scanner and data was processed. All chips passed the quality control assessment. Since these microarray chips contained probe sets for both *S. cerevisiae* and *S. pombe*, the *S. cerevisiae* data was extracted before normalization [22]. There were 5,900 genes for *S. cerevisiae* on the Affymetrix chip. The robust multi-array analysis (RMA) normalization method was used to preprocess the probe level data [23] and expression levels are log base 2 transformed. Quality control assessment and preprocessing of the data were performed using the AffyPLM R package [24] available from Bioconductor (www.bioconductor.org). Data can be accessed at the National Center for Biotechnology (NCBI) Gene Expression Omnibus (GEO) database (GSE74259).

The data were analyzed using the *limma* package [25] from Bioconductor. An absolute value of a log-fold change of at least 2 with an adjusted *p* value less than 0.05 were used as the criteria for up or down regulation of expression. The gene ontology (GO) enrichment tool from the *Saccharomyces* Genome Database (SGD; www.yeastgenome.org) was used to group genes based on biological function.

Reverse transcription-quantitative PCR analysis (RT-QPCR)

DNase I-treated RNA (1µg) was reverse transcribed to cDNA using the Superscript III system (Invitrogen). For all PCR assays, primers were designed using the NCBI databases and PrimerSelect (DNASar) with the following optimal characteristics: melting temperature

= 50–55°C, limited potential primer-dimer or hairpin structures, and final product size between 100–250 base pairs; Basic Local Alignment Search Tool (BLAST) queries were used to verify the specificity of each primer (Table 1).

QPCR was performed using an ABI PRISM 7900 HT Sequence Detection System with collection software SDS2.2 (Applied Biosystems) using SYBR Green to analyze DNA synthesis. “No-RT” controls were included to check for genomic DNA contamination. Cycle threshold (CT) values for 2 replicates were averaged; CT values for tubulin 3 (TUB3) endogenous controls were subtracted from target gene CT values for normalization. Normalized CT values for untreated time zero (the calibrator) were subtracted from all other samples to give a $\Delta\Delta CT$ value. Fold-changes in expression were determined by plotting $2^{-\Delta\Delta CT}$.

RESULTS

To establish yeast as a model system for global gene expression changes due to TFM treatment, growth analyses in culture and viability studies were performed for early-log cultures of *S. cerevisiae* in fermentable and non-fermentable media containing different concentrations of TFM (0.05mM, 0.1mM, and 0.5mM). In fermentable media, growth in TFM-treated cultures was similar to untreated controls [both water-treated (UT-C) and 2% isopropanol-treated (UT)] except for at the highest dose (0.5mM, Figure 1A). When early-log yeast cultures were grown in non-fermentable media containing ethanol and glycerol as carbon substrates for oxidative phosphorylation, all doses of TFM reduced yeast culture density over time as measured by spectrophotometry (Figure 1B). Analysis of colony growth of TFM-treated cultures to determine whether reduced culture density was due to cell death showed that TFM at all doses reduced viability in both fermentable and non-fermentable media; these effects were more pronounced in non-fermentable media (Figure 1C,D). In YPGE, all doses (0.05mM, 0.1mM, and 0.5mM) showed similar effects on viability, and within approximately 24 hours of TFM exposure all cells were dead. These results suggest that, in yeast, TFM uncouples oxidative phosphorylation which compromises ATP production, leaving cells unable to survive long term when forced to metabolize aerobically.

To determine the acute effects of TFM exposure on eukaryotic gene expression, microarray analysis was performed for *S. cerevisiae* cultures exposed to 0.05mM TFM or 2% isopropanol (solvent control) for 1h, 2h, or 4h. Saturated cultures were utilized in this experiment to eliminate potential gene expression changes due to early to mid-log growth stages. Linear Models for Microarray data (*limma*) [26] was used to analyze the data. The number of differentially expressed genes varied when three methods to adjust the *p* value for the multiple testing issue or false discovery rate (FDR) and four different log-fold change criteria were used (Table 2). Using the Bonferroni FDR correction method (the most conservative criterion) and an absolute value of a log-fold change of at least 2 identified 41 genes as differentially expressed between TFM-treated yeast versus controls (Table 2). The Venn diagram (Figure 2) displays the counts of differentially expressed genes between TFM-treated and untreated yeast at each time point (1h, 2h, 4h). Six of the 41 genes (IMA5, HXT1, HXT3, HXT4, MIG2, and YKR075C) were up regulated at both 2h and 4h of TFM exposure; 1 of these 6 genes (IMA5) was also up regulated in response to TFM exposure at

1h (Figure 3; Supplemental Data, Table S1). An additional 34 genes showed altered expression at 4h of TFM exposure of which 21 were up regulated and 13 were down regulated (Supplemental Data, Table S1, Figure S1). When the 41 genes were analyzed using GO enrichment tool from the SGD (Supplemental Data, Table S2), only 3 GO terms were identified: hexose transport (GO:0008645), monosaccharide transport (GO:0015749), and carbohydrate transport (GO:0008643). Five of the 41 genes were identified with these GO terms, including the hexose transporters HXT1, HXT3, HXT4, and HXT8 and MTH1, a regulator of hexose transport expression. Moreover, 4 of the 6 genes that overlapped between time points have functions related to carbohydrate metabolism, including HXT1, HXT3, and HXT4, and the glucosidase IMA5 involved in polysaccharide breakdown. Another gene in the group of 6, YKR075C, has an undetermined GO biological process but is known to be similar to REG1, a protein involved in negative regulation of glucose repressing genes [27]. These results suggest that TFM exposure induces cells to attempt to increase glucose uptake while promoting polysaccharide breakdown, likely due to TFM's effect of uncoupling ATP synthesis from oxidative phosphorylation and forcing cells to utilize anaerobic fermentation.

To validate the microarray data showing an increased expression of genes involved in carbohydrate metabolism, RT-QPCR was performed for the hexose transporters HXT1 and HXT3, 2 of the most highly up regulated genes in the microarray data set (average log-fold changes of 4.0 and 3.3 at 2h and 4h of TFM exposure versus untreated controls, respectively). Both HXT1 and HXT3 expression increased at both 2h and 4h of 0.05mM TFM exposure, as in the microarray experiment. HXT1 expression increased the most, showing greater than 8 and 10-fold increases at 2h and 4h of TFM exposure when compared to untreated controls (Figure 4). These results suggest that cells exposed to TFM attempt to metabolically shift from aerobic respiration to anaerobic ATP production even when no glucose is available, thus increasing expression of hexose transporters to import needed glucose.

DISCUSSION

S. cerevisiae is well-known to be a system in which to model cellular function in higher eukaryotes [20]. The high degree of conservation of cellular functions between yeast and higher eukaryotes has allowed for in-depth analyses of eukaryotic toxicologic responses [28], metabolic stresses [29], and even aspects of human disease [20,30,31]. In addition, yeast is an organism that is easy to maintain and manipulate with a completely annotated genome (SGD) and a library of deletion mutants for each of its approximately 6,000 genes [32]. The availability of *S. cerevisiae* gene chips containing the entire yeast genome to conduct such experiments has allowed the first high throughput investigation of cellular pathways altered with TFM exposure.

Despite the frequent use of TFM as a measure to reduce sea lamprey populations and the reports of mostly sublethal non-target species effects post-treatment, few studies have addressed potential molecular alterations resulting from TFM treatment. Growth was reduced dramatically when cells were forced to utilize aerobic respiration in the presence of TFM, which was expected since TFM is known to be a metabolic uncoupler of electron

transport and ATP synthesis in the mitochondria [12,13]. Perhaps more surprising were the impacts on growth and viability when yeast were grown in fermentable media containing glucose, the preferred carbon source in yeast. This suggests that other molecular and cellular pathways are altered aside from switching from aerobic respiration to anaerobic fermentation. Exposure to uncoupling agents, including TFM and DNP, results not only in diminished ATP synthesis but also enhanced O₂ consumption [13,33]. In turn, elevated O₂ consumption has been shown to increase the production of reactive oxygen species (ROS) [34], which may have been the cause of the reduction in growth and loss of viability, although whether ROS levels are elevated with TFM exposure has not been specifically tested.

Microarray analysis enabled a global investigation of the effects of acute TFM exposure on eukaryotic gene expression over time (1h, 2h, and 4h). Some of the most significantly up regulated genes were related to carbohydrate transport and metabolism. This suggests an attempt to increase ATP production via glucose uptake and/or glycogen breakdown and glycolysis in the presence of TFM. Given that the cells were grown in non-fermentable media for this experiment, it is likely that, despite the acute attempt to increase glucose uptake and glycogen breakdown, longer-term exposure ultimately resulted in the loss of viability that was observed. It has been shown that glycogen stores are depleted in rainbow trout and sea lamprey exposed to TFM [14,15], likely due to the reliance on glucose and anaerobic glycolysis to synthesize ATP. The present study, in which yeast were grown in non-fermentable media, models this condition of low systemic glucose availability, leading to the hypothesis that carbohydrate transporters are up regulated in tissues of organisms exposed to TFM.

Structurally and functionally, TFM is similar to DNP, differing only by a 3-trifluoromethyl versus a 2-nitro group for DNP. Given the similarities between TFM and DNP and that previous microarray studies have been performed on differential gene expression related to DNP exposure, the cellular pathways affected with TFM versus DNP exposure were compared. Microarray studies of DNP exposure in rat hippocampal neuronal cultures [35] and intact mouse cortex [36] revealed an upregulation of genes involved in both cyclic AMP and phosphoinositide 3-kinase signaling, suggesting a neuroprotective role for DNP at low doses. Even with less stringent criteria for identifying differential gene expression in the microarray data (log-fold change of 1 with the Bonferroni correction), the GO enrichment analysis of the 547 differentially expressed genes (Table 2) did not include the aforementioned DNP-altered pathways. In addition, neither microarray study using DNP reported alterations in glucose metabolism. Taken together, this suggests that TFM and DNP may have unique effects on eukaryotic cells, although testing this in the same model system is necessary.

Many of the genes related to carbohydrate transport and metabolism observed to be differentially expressed with TFM exposure were also up regulated in other microarray studies related to glucose sensing pathways in yeast. The glucose sensing regulatory system in yeast is complex and involves several signaling pathways that are interconnected and respond differently to varying glucose availabilities [27]. When glucose concentrations are low, the transcriptional repressor RGT1 inhibits hexose transporter expression; alternatively,

when glucose concentrations are high, a complex signal transduction pathway is activated downstream of the transmembrane glucose sensors SNF3 and RGT2, resulting in a loss of RGT1 repression and subsequent increased expression of specific hexose transporters [27]. In the present study, 4 hexose transport genes (HXT1, HXT3, HXT4, and HXT8) as well as the genes encoding the paralagous proteins MSH1 and STD1 were found to be significantly up regulated over time; all 6 genes are part of the SNF3/RGT2 glucose sensing pathway in fungi [37,38]. Indeed, 9 of the 41 genes identified as up regulated due to TFM treatment were also significantly up regulated when RGT1 is deleted [39]; these genes include HXT1, HXT 3, HXT4, HXT8, MIG2, MTH1, YOR338W, YKRO75C, and YNL234W. In both the present study and RGT1 deletion study [40] the cells were glucose starved; the similarity in gene expression changes suggests that TFM treatment mimics transcriptional activation that normally occurs when high glucose concentrations and loss of RGT1 repression are present. Whether these cellular changes are due indirectly to the metabolic stress caused by TFM or directly to the inhibition or activation of transcription factors remains to be tested.

In summary, the present study represents the first global gene expression profile of eukaryotic cells exposed to TFM. Using conservative criteria in the statistical analysis of the data, carbohydrate transport and metabolism were determined to be the biological processes most altered by TFM exposure. These gene expression data complement previous cellular studies that confirm TFM as a metabolic uncoupler and provide a publicly available time course microarray dataset that can be mined to determine previously unknown molecular effects of TFM exposure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Growth inhibition and reduced viability in *S. cerevisiae* with acute TFM treatment. (A, B) OD₆₀₀ readings are shown for yeast treated with water (UT-C), 2% isopropanol (TFM solvent, UT), 0.05mM, 0.1mM TFM, or 0.5mM TFM continuously from early-log phase in fermentable (YPD; A) and non-fermentable (YPGE; B) media. (C, D) Early-log cultures grown in YPD (C) or YPGE (D) were treated for 1h, 4h, 10h, or 24h with TFM (0.05mM, 0.1mM, or 0.5mM) or solvent (UT; 2% isopropanol). Cells were diluted 10⁻⁵ and smeared

onto YPD plates that were incubated for 48h at 30°C after which colonies were counted.
Data points represent averages of 3 separate trials for all experiments and doses.

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Figure 2.

Counts of differentially expressed genes at each time point in 0.05mM TFM-treated yeast versus controls as measured by microarray analysis. The Venn diagram displays the counts of differentially expressed genes between TFM-treated and untreated yeast (growing in non-fermentable media, YPG) at each time point (1h, 2h, 4h) using the Bonferroni correction for the false discovery rate (FDR) and an absolute value of a log-fold change of at least 2. HXT8 was identified overall as being differentially expressed, however, due to the variability of expression at 2h and 4h, it was not identified as differentially expressed at those time points and is not included in the Venn diagram.

Figure 3.

Microarray expression profiles of genes up regulated at 2h and 4h of 0.05mM TFM exposure. The expression profiles (log base 2) of the 6 genes that were up regulated after 2h of TFM exposure in non-fermentable media (YPGE) are shown. The solid line with the symbol "T" represents the expression profile of each gene for TFM-treated yeast. The dotted line with the symbol "U" represents the expression profile of each gene for untreated yeast.

Figure 4.

HXT1 and HXT3 gene expression is up regulated in TFM-treated yeast. Yeast (BY4741) grown in non-fermentable media (YPGE) were incubated with 2% isopropanol (UT), or 0.05mM TFM for 1h, 2h, or 4h. Fold changes in expression relative to untreated controls at time 0 as measured by RT-QPCR are shown for HXT1 (A) and HXT3 (B). Two independent RT-QPCR experiments were performed for each group and time point. Open and hatched triangles represent the first and second trials for untreated yeast, respectively. Open and hatched squares represent the first and second trials for TFM-treated yeast, respectively.

UT-1 and UT-2: Untreated Trial 1, Untreated Trial 2; TFM-1 and TFM-2: TFM-treated Trial 1, TFM-treated Trial 2.

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Table 1

Primers used for RT-QPCR

Gene Name	Forward Sequence (5'-3')	Reverse Sequence (5'-3')
HXT1	CGTTGACCGTTTTGGCCGTCG	TGGGCCCAGGTAGTAGCGAA
HXT3	TTCCGGTTTGGGTGTTGGTGGT	ACATGGCCGGCTTACCAGTGAA
TUB3	CCTGCGCCTCAATTGTCTACT	TTCCAGGGTGGTATGCGTG

Table 2Count of differentially expressed genes by Multiple Testing Correction and log-fold change^a

Method	log-fold change			
	0.5	1	1.5	2
Benjamini Hochberg	2468	679	146	49
Holm-Bonferroni	1212	560	122	41
Bonferroni	1161	548	121	41

^a A comparison by log-fold change in the expression values between TFM-treated and untreated samples and three commonly used methods to adjust the *p* value to reduce the number of false positives is shown. The adjusted *p* value was less than 0.05 and the absolute value of the log-fold change was at least the values stated.