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Development of a yeast biosensor–biocatalyst for the detection and biodegradation of the organophosphate paraoxon

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Abstract Organophosphate (OP) poisoning can occur through unintentional exposure to OP pesticides, or by the deliberate release of OP nerve agents. Consequently, there is considerable interest in the development of systems that can detect and/or biodegrade these agents. The aim of this study was to generate a prototype fluorescent reporter yeast biosensor that could detect and biodegrade the model OP pesticide, paraoxon, and subsequently detect paraoxon hydrolysis. *Saccharomyces cerevisiae* was engineered to hydrolyze paraoxon through the heterologous expression of the *Flavobacterium* species *opd* (organophosphate degrading) gene. Global transcription profiling was subsequently used to identify yeast genes, which were induced in the presence of paraoxon, and genes, which were associated with paraoxon hydrolysis. Paraoxon-inducible genes and genes associated with paraoxon hydrolysis were identified. Candidate paraoxon-inducible promoters were cloned and fused to the yeast-enhanced green fluorescent protein (yEGFP), and candidate promoters associated with paraoxon hydrolysis were fused to the red fluorescent protein (yDsRed). The ability of the yeast biosensor to detect paraoxon and paraoxon hydrolysis was demonstrated by the specific induction of the fluorescent reporter (yEGFP and yDsRed, respectively). Biosensors responded to paraoxon in a dose- and time-dependent manner, and detection was rapid (15 to 30 min). yDsRed induction occurred only in the recombinant *opd*$^{+}$ strains suggesting that yDsRed induction was strictly associated with paraoxon hydrolysis. Together, these results indicate that the yeast biocatalyst–biosensor can detect and degrade paraoxon and potentially also monitor the decontamination process.

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Introduction

Organophosphate (OP) agents are poisonous chemicals that exert their toxicity by inhibiting the enzyme acetylcholinesterase (AChE; Broomfield and Kirby 2001; Raushel 2002). OP exposure causes respiratory symptoms, chest tightness, dimming of vision, eye pain, and in severe cases, vomiting, abdominal pain, bladder/bowel hyperactivity, convulsions, respiratory failure, paralysis, and death. OP compounds (e.g., paraoxon, parathion, coumaphos, and diazinon) are commonly found in agricultural and domestic pesticides. Over 40 million kilograms of OP pesticides are used annually in the USA, with another 20 million kilograms produced for export. Unintentional pesticide exposure (water, food, and soil) results in 1 to 3 million poisonings annually worldwide.

OP agents such as VX (*O*-ethyl *S*-[2-diisopropylaminoethyl] methylphosphonothiolate) and sarin also pose a threat as chemical warfare agents. Consequently, there is considerable interest in developing self-sustainable detection systems and strategies for disposing of these man-made toxic compounds in both the civilian and military sectors.

OPs can be chemically or enzymatically hydrolyzed (Hoskin et al. 1999; Mulchandani et al. 1999; Raushel 2002; Irvine et al. 2004). Several enzymes from different organisms (e.g., mammals, bacteria, squid, clams, and protozoa) have been found to reduce OP toxicity by hydrolyzing specific classes of pesticides and nerve agents. Of these, the bacterial organophosphorus hydrolase (OPH, E.C. 3.1.8.1) encoded by the *opd* gene (organophosphate-degrading) of *Pseudomonas diminuta* and *Flavobacterium* species, has the broadest activity (Mulbry and Karns 1989; Di Sioudi et al. 1999; Raushel 2002). Consequently, different strategies have incorporated the unique properties of the OPH enzyme into OP agent degradation systems, biosensors, and even human prophylactic treatments (Broomfield 1993; Mulchandani et al. 2001). For example, the purified enzyme has been incorporated into silicon nanocomposites and fire fighting foams for the generation of protective wear and the decontamination of OP-contaminated surfaces, respectively (LeJeune and Russell 1999; Gill and Ballesteros 2000).

Whole cell biocatalysts may be more advantageous for bioremediation strategies in comparison to purified enzymes since intact cells will be easy to generate and cheap to produce in bulk quantities while the purification of enzymes can be tedious, expensive, and be difficult to handle. In addition, the yeast *Saccharomyces cerevisiae* offers advantages over prokaryotic systems since: (1) it is genetically well defined with a plethora of mutants available through the *Saccharomyces* Genome Deletion Project (Stanford); (2) the survival rates after lyophilization and storage are superior than for bacteria, and (3) yeast genotoxic-inducible genes respond to a much broader spectrum of damaging agents than bacteria. *S. cerevisiae* biosensors have been developed for the detection of various agents including DNA damaging compounds, pollutants, and toxic metal contaminants (Billinton et al. 1998; Cahill et al. 2004; Shetty et al. 2004; Radhika et al. 2005). Yeast biosensors typically use green fluorescent protein (GFP) as a read-out signal since this reporter is stable, does not require exogenous substrates or cofactors, accumulates in the cytoplasm, and can be monitored without any sample preparation. In addition, dual reporter biosensors, which make use of GFPs with distinct spectral properties, have been developed to differentially fluoresce in the presence of different stimuli (Ehrig et al. 1995; Ropp et al. 1996; Yang et al. 1996). The aim of this study was to develop a prototype fluorescent yeast biosensor that can detect and biodegrade the model OP agent paraoxon, and differentially detect paraoxon hydrolysis. This yeast biosensor is unique in that it can detect and decontaminate the OP agent, and also potentially monitor the decontamination process.

Materials and methods

Microorganisms and propagation

S. cerevisiae W3031A (MATa *ade2-1 ura3-1 his3-11 trp1-1 leu2-3 leu2-112 can1-100)* and BY4741 (MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*), were grown and maintained on 1% yeast extract, 2% peptone, and 2% dextrose (YPD, Difco) broth or YPD agar at 37°C. Where appropriate, strains were grown in synthetic dropout (SD) media with different carbon sources (2% glucose, 2% galactose, or 1% sucrose, and 3% galactose). The membrane mutant strain *erg6* (ATCC4000568) was maintained on YPD supplemented with 200 μg/ml geneticin. *Escherichia coli* ER2738 (F' $proA^{+}B^{+}$ $lacI^{q}$ *Δ(lacZ)M15 zzf::Tn10(*Tet^{R}*)/fhuA2 glnV Δ(lac-proAB) thi-1 Δ(hsdS-mcrB)5*) was grown in Luria Bertani (LB) broth at 37°C with the appropriate antibiotics as needed.

Yeast OPD vector and transformation

The plasmid pJK33, containing the *Flavobacterium opd* gene lacking the signal peptide sequence, was kindly provided by Dr. Walter Mulbry (Mulbry and Karns 1989). The 1,020 bp *opd* gene was PCR-amplified using pJK33 as template. The 5′ primer was designed to incorporate a preferred sequence upstream of the ATG start codon site to ensure translation initiation in yeast (Looman and Kuivenhoven 1993). In addition, the 5′ and 3′ primers were also designed to incorporate the restriction endonuclease sites *Bam*HI and *Hin*dIII, respectively (Table 1). The *opd* gene was directionally cloned into the corresponding sites of the *S. cerevisiae* expression vector pESC–URA using standard molecular biology techniques (Sambrook et al. 1989) to create pOPD–ESC–URA. This placed the *opd* gene under the transcriptional control of the pGAL1 promoter for repressed (glucose) or induced (galactose) expression in *S. cerevisiae*. Yeast transformations were performed using the LiAc transformation procedure (Ito et al. 1983). Yeast transformants were selected on the appropriate SD medium supplemented with 2% glucose.

Paraoxonase assays

S. cerevisiae W3031A, harboring pESC–URA (empty plasmid control) and pOPD-ESC–URA plasmids, was grown in SD medium containing 2% galactose (pGAL1 inducing conditions, SDgal) at 37°C. At an optical density

Table 1 PCR primers used for gene and promoter clonings

Gene[a]/promoter[b]	Primers (5′-3′)	Gene[a]/promoter[b] fragment size (bp)
Opd[a]	TTTAA**GGATCC**AAAAGTATGACCATGATTACGAATTCC	1020[a]
	TTTAT**AAGCTT**CATGACGCCCGCAAGG-Rev	
yEGFP[a]	TATTA**GGATCC**ATTAAAATGTCTAAAGGTGAAG	724[a]
	TATAA**ACTAGT**TTATTTGTACAATTCATCCATAC-Rev	
YGR035C[b]	TTAA**GTCGAC**AGAGTTTACTTGCACTACC	991[b]
	TTAA**GTCGAC**GGAGATAATACCCTCTG	455[b]
	AATT**GGATCC**TACGTATTGTTTCTGTAGAG-Rev	
YLR346C[b]	AATT**GTCGAC**TTTCTTCCTTTTCTACTATC	973[b]
	TTAA**GTCGAC**AACCATCCTTAGCTTCC	476[b]
	TTAT**GGATCC**TATTAAAGGTGATAAATAATCC-Rev	
POX1[b]	AATT**GTCGAC**TTTCACTCAACCACCTCC	1002[b]
	TATA**GTCGAC**TAGATTCCTTCAGTTCCAC	525[b]
	TTAA**GGATCC**ATCGCAATACTAATTTATTATAT-Rev	
YGR287C[b]	TTAA**GTCGAC**GGCTGTATGTTTATGATTGC	993[b]
	TTAA**GTCGAC**GCTCTTAACTGCGTTTACC	570[b]
	TTAA**GGATCC**GATAGTAAATATTACGTTGA-Rev	

Bold indicates the *Bam*HI (GGATCC), *Hin*dIII (AAGCTT), *Spe*I (ACTAGT), or *Sal*I restriction sites
Rev, reverse primer
[a] Gene
[b] Size of 5′ promoter sequence relative to the ATG start size

$(OD)_{600}$ of approximately 0.2, yeast cells were harvested by centrifugation and enzyme lysates were prepared by incubation with Y-MER dialyzable lysis buffer (Pierce Biotechnology) for 20 min at room temperature (RT), followed by centrifugation at 24,000×*g* for 15 min at 4°C. To measure cytosolic paraoxonase activity, 25 μl of the supernatant was mixed with 975 μl of reaction buffer (50 mM HEPES pH 7.5, 0.5 mM paraoxon). For the TRITON extractable enzyme assay (membrane associated), the membrane pellet was resuspended in 100 μl of 1% TRITON X 100 in 50 mM HEPES pH 7.5, incubated for 20 min at RT, and centrifuged for 2 min at 24,000×*g*. The resulting supernatant was tested for paraoxonase activity. *P*-nitrophenol release was measured at 405 nm with 3 replicates for each triplicate sample. Paraoxonase activity was calculated using the extraction coefficient of 1.7×10^4 $M^{-1}cm^{-1}$ and presented as μmol paraoxon hydrolyzed/min/mg protein. Whole cell yeast assays were as above except yeast cells were assayed directly in the reaction buffer. Activity is presented as μmol paraoxon hydrolyzed/min/10^9 colony forming units (CFU).

RNA preparation

Exponentially growing *S. cerevisiae* W3031A cultures, harboring either pESC–URA (control strain) or pOPD–ESC–URA (OPH^+ strain), were incubated at 37°C in SDgal in the presence or absence or 3 mM paraoxon. After 60 min incubation, the cultures (OD_{600} of approximately 0.4) were harvested and disrupted by continuous vortexing in TRI reagent in the presence of glass beads (425–600 μm) for 10 min, and RNA was extracted according to the manufacturers' recommendations (Ambion). RNA preparations were DNase1-treated and purified using RNeasy columns (Qiagen).

Microarray analysis

Biotin-labeled, fragmented cRNA targets were prepared from 10 μg of total RNA using standard protocols established for the Affymetrix GeneChip System. Targets were hybridized to the Affymetrix GeneChip Yeast Genome S98 array, which contains the entire *S. cerevisiae* genome (approximately 6,400 ORFs) on a single array. Hybridizations were performed in triplicate using three independent RNA preparations. Post-hybridization washing, staining, and scanning were performed using standard conditions developed by Affymetrix. Microarray hybridization data (CEL files) were normalized using the Bioconductor implementation of GCRMA (Gentleman et al. 2004), and normalized hybridization data were imported into the analysis program dChip for pairwise comparison of treatments (Cheng and Wong 2003). Paraoxon-induced genes were defined by the following criteria: increase in expression greater than fourfold and statistical difference (unpaired *t* test, $p<0.01$) for wild-type paraoxon cultures versus control and recombinant OPH^+ paraoxon cultures versus control. Candidate genes associated with paraoxon hydrolysis were defined by the following criteria: increase in expression greater than

twofold and statistical difference (unpaired *t* test, $p<0.01$) for OPH^+ paraoxon cultures versus OPH^+ control excluding paraoxon-induced genes.

Real-time and relative RT-PCR

Equal amounts of RNA (~1.5 μg) were reverse transcribed into cDNA using the retroscript kit (Ambion). Real-time primers were designed to have a Tm between 58 and 60°C and an amplicon size of 80–200 bp (Table 2). Reactions were run in duplicate using the BioRad iCycler iQ real-time PCR detection system and the iQ SYBR Green qPCR SuperMix (BioRad). All primer pairs produced a single amplicon with a uniform melting curve as determined by the dissociation profile of the product. For all samples, the gene of interest input quantity was determined using a relative standard curve and normalized with the input quantity of the endogenous reference (actin, *ACT1*). The normalized amount of target was then divided by the calibrator (untreated control) and values were expressed as a *n*-fold change.

Relative RT-PCR conditions were as follows; initial denaturation at 95°C for 2 min, and then 25 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s, with a final extension at 72°C for 10 min. To check for the presence of genomic DNA, reactions were performed using both the relative (Table 2) and real-time PCR primers with samples lacking the addition of reverse transcriptase; PCR products were not obtained from these control reactions (data not shown).

Table 2 Real-time and relative RT-PCR primers used in this study

Gene/ORF	Primers (forward and reverse, 5′-3′)	PCR product size (bp)
YGR035C[a]	GCGTGCCATAGTTTCTTCC	169
	TCGTCCCTGAGACTTACTC	
YGR035C[b]	CAGCCAAGACTACAAGAACG	93
	GGGAAGAAACTATGGCACG	
YLR346C[a]	TGTCCCATCGGGTTAGTTTC	161
	TGGGCAGCCTTGAGTAAATC	
YLR346C[b]	GCAGAGTGGGTAGCATGTC	131
	AACTCCTGCATTTCCGCTTC	
YOR186W[a]	TTTAAGTCCGCCATACGAGC	139
	GCTGCAACCTGAATTCTTCC	
SPS100[b]	AGTACCGTGGCATCATGGAC	128
	GCAGAAATTGCACCTTGTGG	
RTA1[b]	AATGGCTACTCTGCTGTTCG	87
	CTGCCTACATCGCCAATCAC	
MET28[b]	AAGCAGGAAGGGTATGAACG	100
	CAGCAGCAATGATAGCAAGC	
YLL056C[b]	CAGCAGCAATGATAGCAAGC	189
	CGGTCAATTTCGCAGCACTG	
POX1[a]	AAGCTGCTCTGGTTACCGTG	134
	ATACCCATGTCCTCCGCAAG	
YGR287C[a]	ACTTGGCAATCCAGTGATCC	140
	TCGGAGGCATGTTGCATTTC	
HXT9[a]	AAGTTGTGGCCTCAAGGAAG	183
	TGTTGCGATTGCCATTCCTC	
YHL012W[a]	TTGCCTTCTTTCCGAACAGT	173
	GCCCAAGTCGATTGAAGGTA	
ACT1[a]	TTCCAGCCTTCTACGTTTCC	100
	ACGTGAGTAACACCATCACC	
ACT1[b]	TCCTACGTTGGTGATGAAGC	547
	GAAGATTGAGCAGCGGTTTG	

[a] Real-time RT-PCR primers
[b] Relative RT-PCR primers

yEGFP and yDsRed promoter fusions

A *Candida albicans* strain containing yEGFP was kindly provided by Dr. Edward Balish (Medical University of South Carolina). The 700 bp yEGFP gene was PCR-amplified using *C. albicans* yEGFP genomic DNA as template, and primers incorporating *Bam*HI and *Spe*I restriction sites (Table 1). Cloning of the *Bam*HI/*Spe*I digested yEGFP PCR product into the corresponding sites of pESC–HIS (Stratagene), dropped-out the pGAL10 and pGAL1 plasmid promoters, to create a 'promoterless' pyEGFP–HIS plasmid. The yeast codon-optimized DsRed-express gene (Clontech) was generated by Bio S and T (Montreal, Canada) and cloned into the *Bam*HI/*Not*I sites of pESC–LEU (Stratagene) to create a 'promoterless' pyDsRed–LEU plasmid.

Promoter fragments for the paraoxon-inducible genes (YGR035C, YLR346C) and the genes associated with paraoxon hydrolysis (*POX1*, YGR287C) encompassing approximately 500 and 1,000 bp upstream of the ATG start site were PCR amplified from *S. cerevisiae* W3031A genomic DNA. The 5′ and 3′ primers incorporated *Sal*I and *Bam*HI sites, respectively (Table 1). The YGR035C and YLR346C promoter fragments were cloned into the *Sal*I/*Bam*HI of pyEGFP–HIS, thereby placing yEGFP under the transcriptional control of the paraoxon-inducible promoters. The same strategy was utilized for cloning *POX1* and YGR287C promoter fragments into pyDsRed–LEU to generate a yDsRed reporter under the control of promoters associated with paraoxon hydrolysis.

Fluorescence assays

S. cerevisiae BY4741 wild-type and recombinant OPH^+ were transformed with the promoter-reporter constructs and the empty 'promoterless' control vectors. *S. cerevisiae* BY4741 was used in preference to *S. cerevisiae* W3031A since W3031A exhibited higher autofluorescence than BY4741. For example, using the FLUOstar OPTIMA plate reader (BMG Lab Technologies), W3031A exhibited 2.6 and 7.5-fold more autofluorescence for GFP (excitation and emission

Table 3 Paraoxonase activity of yeast OPH^+ enzyme lysates and intact cells

Source	Paraoxonase activity[a]		
	Cytosolic fraction	TRITON extractable	Intact cells
Enzyme lysate[b]	0.21 (0.11)	0.64 (0.12)	NA
Whole cell[c]	NA	NA	0.33 (0.12)

[a] Average activity of triplicate samples ± (standard deviation) after subtraction for background activity (cultures harboring empty plasmid)
[b] μmol paraoxon hydrolyzed/min/mg total protein
[c] μmol paraoxon hydrolyzed/min/10^9 CFU

of 485 and 520 nm), and DsRed (excitation and emission of 544 and 590 nm), respectively, than BY4741. Cells were grown in SDgal/suc (3% galactose, 1% sucrose) lacking the appropriate amino acid(s) and early-exponential cells were incubated in the presence or absence of paraoxon for the designated time periods at 37°C. Cells were harvested by centrifugation, washed in phosphate buffered saline, resuspended in 10 mM Tris–HCl pH 8.5, and duplicate samples were measured for GFP or DsRed. Results were normalized to the absorbance (OD_{600}) of the culture, and to the 'promoterless' control vector (pyEGFP–HIS or pyDsRed–LEU). Relative fluorescence is presented as fold-induction compared to cells not exposed to paraoxon.

The abbreviations used are: OP, organophosphate; *opd*, organophosphate degrading gene; yEGFP, yeast enhanced green fluorescent protein; yDsRed, red fluorescent protein codon-optimized for expression in yeast; OPH, organophosphorus hydrolase; CFU, colony forming units.

Results

Functional OPH expression in yeast

S. cerevisiae was transformed with the *opd* expression plasmid and the empty control plasmid. Relative RT-PCR analysis was used to confirm *opd* mRNA expression (data not shown) and paraoxonase assays were performed to determine whether the heterologous expression of the bacterial *opd* gene resulted in active OPH enzyme. Enzyme lysates prepared from OPH^+ yeast cells were capable of hydrolyzing paraoxon (Table 3). Paraoxonase activity from control enzyme lysates (prepared from yeast cells harboring an empty plasmid) was negligible. In addition, no difference in paraoxonase activity was detected in lysates prepared from yeast cells harboring the wild-type *opd* gene or the yeast codon-optimized *opd* gene (data not shown) suggesting that codon bias was not a limiting determinant for efficient expression. Intact OPH^+ yeast cells were also able to hydrolyze paraoxon which demonstrated in principal, that yeast cells were able to function as a biocatalyst (Table 3). Yeast culture supernatants did not exhibit detectable paraoxonase activity. This indicated that OPH was restricted to an intracellular location and paraoxon hydrolysis occurred within the cell. For paraoxon to gain entry into the cell, it must pass through both the yeast cell wall and cell membrane. Ergosterol is the principal sterol found in yeast membranes and mutations in ergosterol biosynthesis have been shown to change membrane fluidity and increase membrane permeability (Gaber et al. 1989; Welihinda et al. 1994; Hemenway and Heitman 1996). To investigate whether a mutation in ergosterol biosynthesis rendered the yeast cell hypersensitive to paraoxon, growth curves were performed for the membrane mutant strain *erg6* in the presence or absence of paraoxon. No difference was observed for the growth of wild-type (BY4741) in the presence of 0.5 mM paraoxon. In contrast, 0.5 mM paraoxon had a dramatic effect on *erg6* growth (Fig. 1). This indicated that a mutation in *ERG6* conferred increased sensitivity to paraoxon, which was most likely due to the increased rates of paraoxon diffusion through the altered yeast membrane.

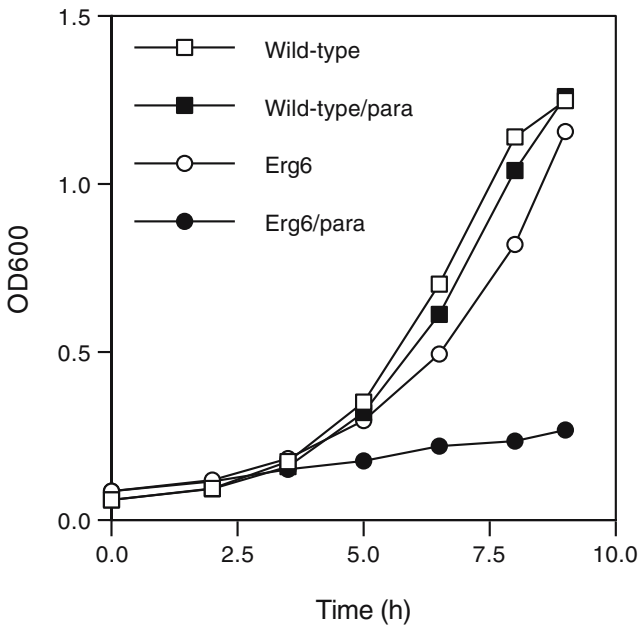

Fig. 1 Effect of paraoxon on the growth of *S. cerevisiae* wild-type (BY4741) and membrane mutant strain *erg6*. Overnight saturated cultures were diluted 1:100 in fresh YPD medium or YPD plus 200 μg/ml geneticin (*erg6*) and grown at 37°C in the presence or absence of 0.5 mM paraoxon (*para*). Growth was monitored spectrophotometrically at OD_{600}

Microarray analysis

To construct a reporter yeast biosensor that responded to the presence of OP agents, paraoxon-responsive promoters must first be identified. The global transcriptional response of *S. cerevisiae* to an OP agent such as paraoxon has not been previously analyzed. Paraoxon-inducible genes were identified by analyzing (microarray) samples prepared from wild-type *S. cerevisiae* incubated in the presence or absence of paraoxon. Microarray analyses was also performed on samples prepared from recombinant OPH^+ yeast in the presence or absence of paraoxon. This was predicted to result in a transcriptional profile that was distinct from cells lacking the ability to hydrolyze paraoxon and enable the identification of genes associated with paraoxon hydrolysis and/or the identification of genes induced by the hydrolyzed products. Biotin-labeled, fragmented cRNA samples, prepared from three independent cultures, were hybridized to the Affymetrix Yeast Genome S98 Array. Of the ~6,400 ORFs, ~1% displayed a fourfold ($p<0.01$) or greater increase in response to paraoxon in both wild-type and OPH^+ yeast; seven of the paraoxon-inducible genes were increased by 50-fold or greater, with the greatest increase being 1,789-fold (Table 4 and supplementary material S1). The number of differentially induced genes associated with paraoxon hydrolysis (OPH^+ paraoxon-inducible genes excluding genes induced by paraoxon in the absence of OPH^+) was not as dramatic. Nevertheless, 32 differentially expressed genes (≥2-fold, $p<0.01$) were identified, of which 6 were induced by at least threefold (Table 5 and supplementary material S2).

Target prioritization

To verify that the genes identified by the microarray analysis were differentially expressed, seven paraoxon-inducible genes and four genes associated with paraoxon hydrolysis were individually analyzed by an independent method. The seven paraoxon-inducible genes (YGR035C, YLR346C, *SPS100*, YOR186W, *RTA1*, *MET28*, and YLL056C) were analyzed initially by relative RT-PCR; the expression of all seven genes increased in response to paraoxon exposure (3 mM paraoxon for 1 h; Fig. 2). Select candidate genes were analyzed by relative and real-time RT-PCR after exposure to 0.05, 0.1, 0.25, 0.5, and 1.0 mM paraoxon. Relative RT-PCR indicated that there were no obvious changes in gene expression for YOR186W, *SPS100*, and *RTA1* at the lower paraoxon concentrations tested (0.05–1.0 mM, Fig. 3a). In contrast, YGR035C was induced 18- and 31-fold by 0.5 and 1.0 mM paraoxon, respectively. Similarly, YLR346C was induced eight- and tenfold by 0.5 and 1.0 mM paraoxon, respectively (Fig. 3b). To examine the temporal induction of YGR035C and YLR346C, *S. cerevisiae* was exposed to 2.5 mM paraoxon for 7.5, 15, 30, and 60 min. Both YGR035C and YLR345C were significantly and maximally (98- and 32-fold, respectively) induced after 7.5 min exposure to paraoxon (Fig. 4). Since YGR035C and YLR346C responded to low paraoxon concentrations, and were also quickly induced, both genes were prioritized for paraoxon-sensitive reporter construct development.

Four candidates (YHL012W, *HXT9*, *POX1*, and YGR287C) associated with paraoxon hydrolysis were verified for differential expression. Real-time RT-PCR was used to examine candidate expression from control yeast (harboring the empty control plasmid) in the absence or presence of paraoxon (C and CP, respectively), and recombinant OPH^+ yeast in the absence or presence of paraoxon (T and TP, respectively). YHL012 expression did not change in the presence or absence of paraoxon, or between the wild-type and recombinant OPH^+ yeast (Table 6). *HXT9* expression was induced approximately threefold by paraoxon; however, paraoxon-induced expression occurred in both wild-type and recombinant OPH^+ yeast cells indicating that *HXT9* induction was not associated with OPH and hence paraoxon hydrolysis. In

Table 4 Partial list of ORFs whose transcripts are induced ≥4-fold by paraoxon in both control and OPH^+ yeast[a]

ORF	Gene	Fold-induction	Function/gene product/description[b]
YGR035C	–	1,789.2	Function unknown; activated by Yrm1p and Yrr1p along with genes involved in MDR
YHR139C	*SPS100*	719.1	Sporulation-specific cell wall maturation protein
YOR049C	*RSB1*	580.1	Phospholipid translocating ATPase activity
YOR186W		269.1	Hypothetical protein
YGR213C	*RTA1*	152.2	Protein involved in 7-aminocholesterol resistance
YGR236C	*SPG1*	106.2	Protein required for survival at high temperature during stationary phase
YLR346C	–	69.6	Unknown function; regulated by transcription factors involved in PDR
YGR066C	–	28.8	Hypothetical protein
YIR017C	*MET28*	27.7	Transcriptional activator. Participates in sulfur metabolism. Stress response
YIL160C	*POT1/FOX3/POX3*	27.3	3-ketoacyl-CoA thiolase with broad chain length specificity

[a] For the full list of ORFs ($n=64$), see supplementary material S1
[b] http://www.yeastgenome.org

Table 5 Partial list of ORFs whose transcripts are induced ≥2-fold by paraoxon in OPH$^+$ yeast but not in control yeast (harboring the empty plasmid control) and excluding ORFs induced solely by paraoxon

ORF	Gene	Fold-induction	Function/gene product/description[b]
YGL205W	*POX1/FOX1*	7.0	Acyl coenzyme A oxidase
YJL219W	*HXT9*	3.9	Putative hexose transporter
YGR287C	–	3.9	Hydrolase
YHL012W	–	3.8	Unknown/similar to UDP glucose pyrophosphorylase
YDL243C	*AAD4*	3.3	Aryl-alcohol dehydrogenase
YAR023C	–	3.1	Unknown
YDR530C	*APA2*	2.9	Tetraphosphatase
YGR067C		2.9	Unknown/similarity to transcription factor
YBR285W	–	2.7	Unknown
YHR124W	*NDT80*	2.7	Transcription factor

[a] For the full list of ORFs (n=32), see supplementary material S2
[b] http://www.yeastgenome.org

contrast, *POX1* expression was induced (2.4-fold) only in recombinant OPH$^+$ yeast in the presence of paraoxon. Similarly, YGR287C expression was increased ninefold in recombinant OPH$^+$ yeast cells in the presence of paraoxon; however, paraoxon also induced YGR287C in control samples (lacking OPH), albeit to a lesser extent (twofold). These results confirmed that *POX1* and YGR287C were expressed preferentially in recombinant OPH$^+$ yeast in the presence of paraoxon, suggesting induction was associated with paraoxon hydrolysis. Therefore, *POX1* and YGR287C promoters were prioritized for biosensor development.

Reporter fluorescence assays

The 'promoters' of two paraoxon-inducible genes (pYGR035C and pYLR346C) were cloned in front of the promoterless yEGFP and two 'promoters' from genes associated with paraoxon hydrolysis (pYGR287C and p*POX1*) were cloned upstream of the promoterless yDsRed. Since the promoters for these genes have not been characterized previously, the promoters were defined by arbitrary sequence 5′ of the ATG start site. To incorporate the necessary elements conferring both promoter activity and paraoxon sensitivity, two promoter fragments for each gene encompassing approximately 500 and 1,000 bp were cloned in front of each reporter gene (Table 1).

yEGFP assays were performed to analyze whether the yeast biosensor harboring the paraoxon-inducible yEGFP reporter constructs responded to the presence of paraoxon.

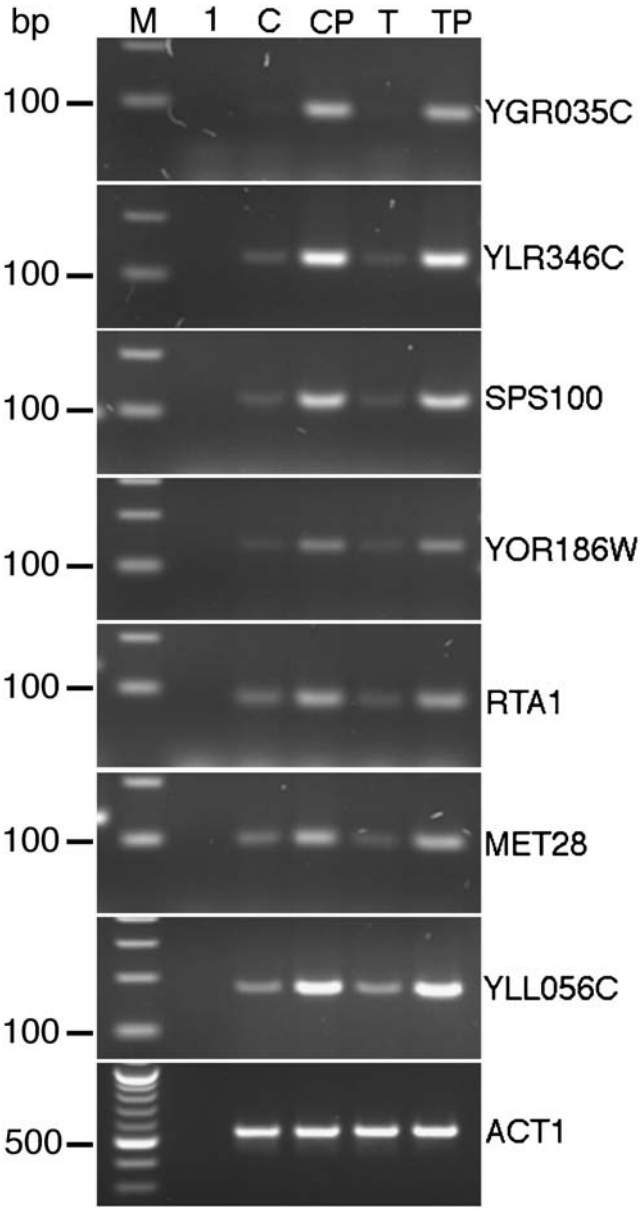

Fig. 2 Confirmation of differential expression of candidate paraoxon-inducible genes using relative RT-PCR analysis. RNA was prepared from control (*C*) or OPH$^+$ (*T*) yeast incubated in the absence (*C*, *T*) or presence of 3 mM paraoxon for 60 min (*CP*, *TP*). PCR reaction performed in the absence of cDNA template (*lane 1*). *M*, 100 bp DNA ladder

Fluorescence assays were performed initially to determine whether the larger (~1,000 bp) or smaller (~500 bp) YGR035C and YLR346C promoter fragments were preferable for conferring both expression and paraoxon sensitivity; preliminary assays indicated similar fluorescence intensity and fold-induction irrespective of the size of the putative promoter; however, the YLR346C promoter fragment elicited a greater response to paraoxon than the YGR035C promoter fragment (data not shown). Therefore, the following experiments were performed with the larger YLR346C promoter

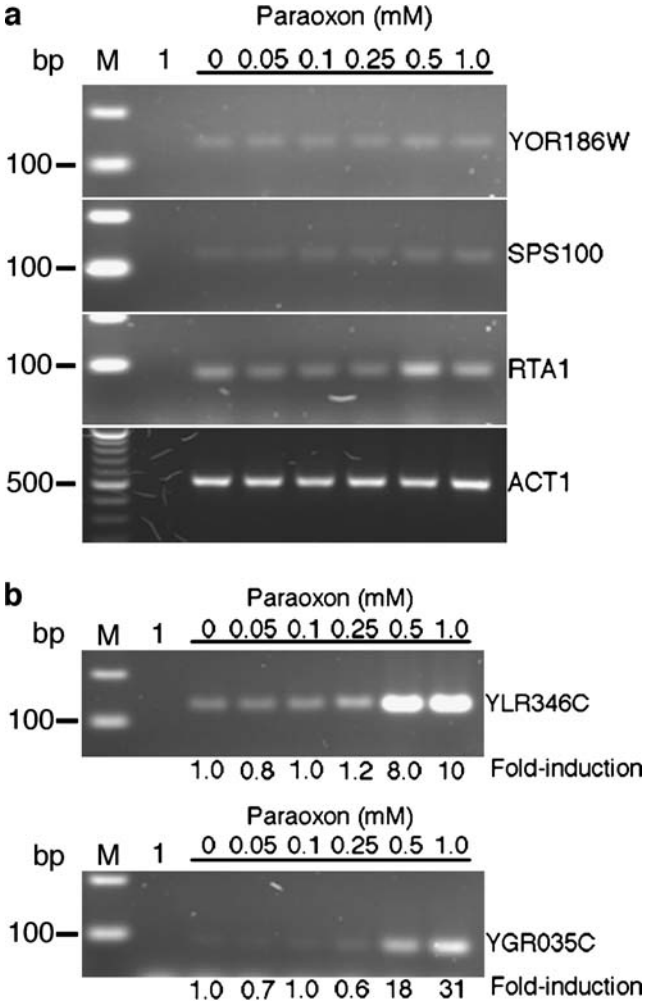

Fig. 3 **a** and **b** Dose-dependent changes in candidate paraoxon-inducible genes using relative and real-time RT-PCR analysis in response to different paraoxon concentrations. RNA was prepared from yeast incubated with varying concentrations of paraoxon for 60 min. Relative RT-PCR analysis was used to discern changes in gene expression (**a** and **b**) while real-time RT-PCR was used to quantitate changes in the prioritized YLR346C and YGR035C candidates (**b**). The numbers below the figure represent fold-induction relative to the control sample as determined by real-time RT-PCR. PCR reaction performed in the absence of cDNA template (*lane 1*). *M*, 100 bp DNA ladder

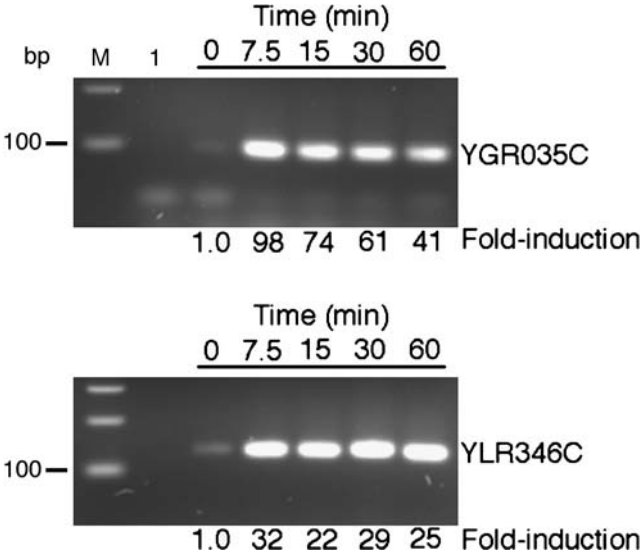

Fig. 4 Time course of YGR035C and YLR346C induction using relative and real-time RT-PCR analysis. RNA was prepared at the indicated times from yeast incubated with 2.5 mM paraoxon. The numbers below the figure represent fold-induction relative to the control sample as determined by real-time RT-PCR. PCR reaction was performed in the absence of cDNA template (*lane 1*). *M*, 100 bp DNA ladder

fragment only. A time course analysis of yEGFP induction was performed with yeast incubated in the presence or absence of 3-mM paraoxon. Yeast cells harboring pYLR346C-yEGFP exhibited increased fluorescence in the presence of paraoxon; yEGFP fluorescence was induced up to 5.1-fold after a 2 h incubation with paraoxon (Fig. 5a). Induction was rapid with GFP increasing approximately twofold after only 15 min exposure to paraoxon. Exposure to paraoxon for periods longer than 2 h (4, 6, 8, and 24 h) did not increase the level of induction. To determine whether the recombinant yeast responded to different paraoxon concentrations, yeast cells harboring pYLR346C–yEGFP were incubated with 0.1, 0.25, 0.5, 1.0, and 3 mM paraoxon, and GFP fluorescence was measured. As the concentration of paraoxon increased, the level of GFP induction increased (Fig. 5b). In addition, fluorescence induction was detected, albeit modestly (1.3-fold), at the lowest concentration of paraoxon tested (0.1 mM).

To analyze whether the pPOX or pYGR287C–YDsRed constructs were 'switched on' by paraoxon in OPH$^+$ recombinant yeast, YDsRed fluorescence assays were performed. The rationale for using DsRed was that paraoxon detection (GFP fluorescence) and paraoxon hydrolysis (DsRed fluorescence) could potentially be measured in a single cell since each protein emits distinct spectral properties; however, due to the poor segregational characteristics of 2μ plasmids, it was not practical to simultaneously measure fluorescence from a cell

Table 6 Real-time RT-PCR analysis of the candidate genes associated with paraoxon hydrolysis

Accession/name	Fold-induction[a]			
	Control[b]	Control/P[c]	OPH$^+$[d]	OPH$^+$/P[e]
YHL012W	1.0	0.8	1.0	1.0
HXT9	1.0	2.8	1.0	2.7
POX1	1.0	0.9	1.0	2.4
YGR287C	1.0	1.9	1.0	9.1

[a] Relative induction (paraoxon compared to no paraoxon)
[b] Control yeast harboring the empty vector
[c] Control yeast incubated with 3 mM paraoxon (P) for 1 h
[d] OPH$^+$ yeast
[e] OPH$^+$ yeast incubated with 3 mM paraoxon (P) for 1 h

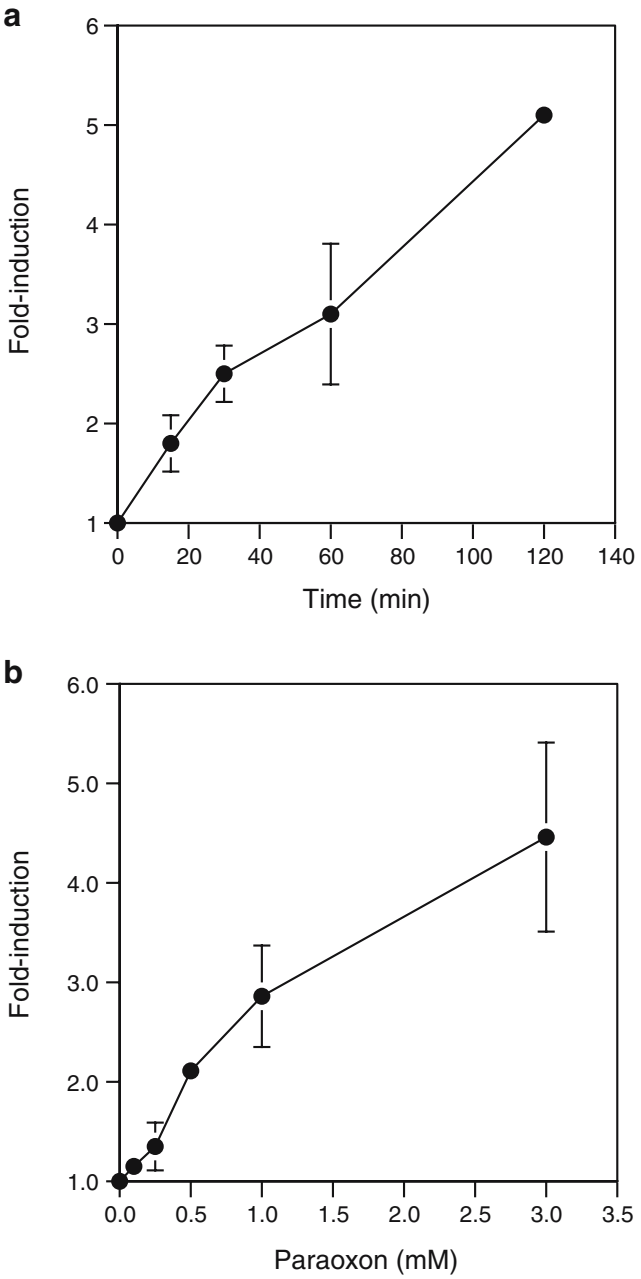

Fig. 5 **a** Time course and **b** dose response to paraoxon. *S. cerevisiae* BY4741 cultures harboring pYLR346C–yEGFP were divided equally and incubated in the absence or presence of 3 mM paraoxon for 15, 30, 60 and 120 min (**a**), or with varying concentrations of paraoxon for 120 min (**b**). Fold-induction represents the average±SD of three independent experiments

harboring three episomal plasmids (OPH$^+$, DsRed$^+$, GFP$^+$ plasmids). *S. cerevisiae* BY4741 harboring pESC–URA (control strain lacking OPH) was transformed with the promoter-YDsRed constructs or the empty 'promoterless' control vector (pYDsRed–LEU). The recombinant OPH$^+$ (test strain, expressing OPH) was transformed with the analogous plasmids. Recombinant OPH$^+$ cells, incubated in the presence of paraoxon, demonstrated induced YDsRed fluorescence compared to cells lacking paraoxon for the four constructs analyzed (Table 7); Induction ranged from two- to threefold, and was similar to the induction results obtained with quantitative real-time RT-PCR. Importantly, the same constructs were not induced by paraoxon in the control strain lacking OPH. Therefore, YDsRed was not induced by paraoxon per se; induction required OPH suggesting that YDsRed induction was strictly associated with paraoxon hydrolysis.

YDsRed induction was observed for the pPOX1–F1–YDsRed fusion construct in a time-dependent manner (Fig. 6a); DsRed-induction increased as the exposure time to paraoxon increased with a maximal induction of 2.5-fold after 260 min. To investigate whether the recombinant yeast responded to different paraoxon concentrations, *S. cerevisiae* OPH$^+$ harboring pPOX1–F1–YDsRed was incubated with 0.1, 0.25, 0.5, 1.0, and 3 mM paraoxon for 270 min before assaying for DsRed fluorescence. As the concentration of paraoxon increased to 1 mM, YDsRed-induction increased, albeit modestly (Fig. 6b). Therefore, the results suggested that the yeast biosensor exhibited a dose response that was characteristic of paraoxon hydrolysis.

Discussion

Yeast cells are ideal for OP biosensor–biocatalyst development since: (1) they are fairly resistant to environmental extremes; (2) they are genetically well defined, with a plethora of mutants available through the *Saccharomyces* Genome Deletion Project; (3) they have been used extensively for the production of functional heterologous proteins including the human organophosphoric acid anhydrolase (OPAA; Wang et al. 2004), and (4) they exhibit good survival rates after long-term storage (Diniz-Mendes et al. 1999; Lodato et al. 1999; Miyamoto-Shinohara et al. 2000).

Table 7 YDsRed fold-induction of *S. cerevisiae* BY4741 control or OPH$^+$ cells harboring the promoter-YDsRed constructs

Promoter[c]	Fold-induction[a,b]	
	Control yeast	OPH$^+$ yeast
pYGR287C-993	1.05 (0.31)	2.56 (0.44)
pYGR287C-570	0.81 (0.28)	2.51 (0.08)
pPOX1-1002	0.89 (0.05)	2.80 (0.18)
pPOX1-525	0.56 (0.04)	2.52 (0.90)

[a] YDsRed fold-induction in response to 3 mM paraoxon for 4.5 h relative to the 'promoterless' control vector and normalized to OD_{600}
[b] Average±(standard deviation) of three independent cultures
[c] Refers to the ORF or gene promoter and the size of the putative promoter fragment

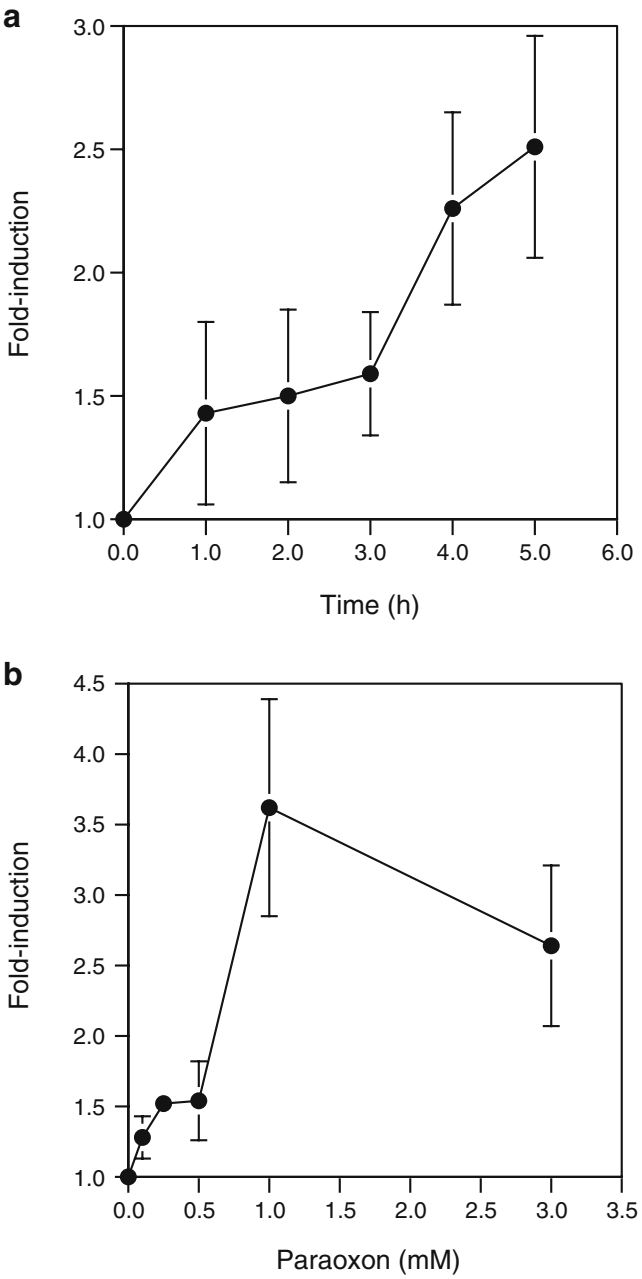

Fig. 6 **a** Time course and **b** dose response to paraoxon. *S. cerevisiae* BY4741 OPH$^+$ cultures harboring pPOX1–F1–yDsRed were divided equally and incubated in the absence or presence of 3 mM paraoxon for 1, 2, 3, 4 and 5 h (**a**), or with varying concentrations of paraoxon for 270 min (**b**). Fold-induction represents the average±SD of three independent experiments

Previous studies expressing OPH in the fungus *Gliocladium virens* and OPAA in *S. cerevisiae* also produced a functional protein capable of hydrolyzing its OP substrate (Dave et al. 1994; Wang et al. 2004); however, expression of the OPAA enzyme in yeast yielded enzyme activity that was only 2.5 times higher than control lysates, due to a high intrinsic background activity (Wang et al. 2004). The majority of the enzyme activity (75%) described in this paper was TRITON-extractable suggesting that the OPH enzyme was membrane associated which is similar to the wild-type *Flavobacterium* enzyme (Mulbry and Karns 1989). For paraoxon to be hydrolyzed within the cell, it must pass through both the yeast cell wall and the cell membrane. The rate-limiting step of bacterial whole cell biocatalysts is usually the rate of passive diffusion of the substrate since the bacterial cell envelope acts as a permeability barrier (Hung and Liao 1996; Ni and Chen 2004). Permeabilizing the outer membrane with solvents or using freeze/thaw techniques can increase the rate of passive diffusion, but these procedures usually kill the cell and thus eliminate a major benefit of intact biocatalysts. To overcome this limitation, membrane permeable mutants may be used. A mutation in *ERG6* conferred increased sensitivity to paraoxon, most likely due to the increased rates of paraoxon diffusion through the altered yeast membrane. Therefore, future experiments may be performed with *erg6* strains to increase the rate of OP entry into the cell, and potentially increase the rate OP hydrolysis.

The global transcriptional response of *S. cerevisiae* to OP agents has not been previously analyzed; however, OP pesticides are genotoxic to *S. cerevisiae* and result in gene reversion, gene conversion, and frameshift mutations (Vlckova et al. 1993; Bianchi et al. 1994). The number of genes differentially regulated by paraoxon (1% of the genome, fourfold or greater) and the degree of differential expression (100-fold) was substantial. As expected, the transcriptional response was similar to alkylating agents such as methyl methanesulfonate which has been shown to induce 325 transcripts, including many types of defense mechanisms such as DNA repair and recombination, stress response and detoxification, and cell cycle checkpoints (Jelinsky and Samson 1999).

Yeast cells harboring the paraoxon-responsive yEGFP constructs displayed paraoxon dose-dependent induced fluorescence. The level of induction (up to 5-fold) was significantly less than the transcriptional induction observed with the microarray and real-time PCR data. This discrepancy may be due to: (1) a suboptimal promoter fragment driving yEGFP expression, and (2) insufficient levels of positive-regulatory transcription factor(s) required for inducing expression from promoters located on multi-copy plasmids. Consequently, the endogenous transcription factors required for paraoxon induction may be saturated in the presence of multiple transcription factor binding sites. Plasmid copy number reduction or coexpression of the required transcription factors (if known) may overcome this limitation. The prototype yeast biosensor paraoxon sensitivity was also orders of magnitude lower than previously described organophosphate sensors, which are capable of detecting in the nM range (Halamek et al. 2005; Lei et al. 2005; Liu and Lin 2006). Although improvements in the

sensitivity may be obtained by, for example, using optimal promoter fragments, using a more sensitive reporter system, increasing substrate permeability, and decreasing substrate efflux, it is unlikely that the yeast biosensor will achieve this level of sensitivity. Nevertheless, the yeast system potentially offers the ability to detect and decontaminate the OP agent, and also monitor the decontamination process which makes it unique from other biosensors. It should be noted however, that paraoxon hydrolysis results in the production of para-nitrophenol, which although significantly less toxic than paraoxon, has been shown to cause blood disorders and skin irritation in animal studies exposed to moderately high levels of nitrophenols. Therefore, additional strategies may be necessary to complete the bioremediation process.

Microarray analysis of OPH^+ yeast incubated in the presence of paraoxon also identified yeast ORFs putatively associated with paraoxon hydrolysis based on the criterion of: (1) ORFs only being induced in the presence of OPH and paraoxon; (2) exclusion of ORFs induced solely by paraoxon, and (3) exclusion of ORFs induced by the heterologous expression of OPH. The fact that fewer genes associated with paraoxon hydrolysis were identified was not unexpected since: (1) it was an indirect reaction, relying on the endogenous enzymatic hydrolysis of paraoxon to cause a shift in the transcriptional response; (2) a single exposure time point and paraoxon concentration was used which may not have been optimal for distinguishing paraoxon-inducible genes from genes associated with paraoxon hydrolysis, and (3) the yeast OPH^+ population was not clonal due to the inherent instability of the 2μ plasmids (Murray and Szostak 1983); therefore, a portion of the yeast population may have been unable to hydrolyze paraoxon. Exposing yeast cells directly to the hydrolyzed product may provide a viable alternative for the identification of genes induced by the biodegraded products. Nevertheless, when the promoters from the candidate ORFs (*POX1*, YGR287C) were fused to YDsRed, fluorescence induction was detected only in OPH^+ cells in the presence of paraoxon, suggesting a mechanism whereby the biodegradation of paraoxon can be detected. Although, the correlation between YDsRed fluorescence and the amount of paraoxon hydrolyzed cannot be estimated from this study, OPH^+ yeast cells were able to hydrolyze 0.33 μmol paraoxon/min/10^9 CFU. Therefore, the proof of principal results suggests the ability to detect OP biodegradation.

Although OPH hydrolyzes, and thereby reduces the toxicity of a wide variety of OP pesticides and chemical warfare agents by cleaving the P–O, P–F, and P–S containing bonds, OPH cleaves these bonds with different efficiencies. For example, OPH catalyses the P–O bond of paraoxon very efficiently but catalyses the P–S bond of VX at much lower efficiencies (Di Sioudi et al. 1999; Gopal et al. 2000). In order to improve the catalytic efficiency and specificity of OPH, OPH variants have been generated by designed and random approaches (Watkins et al. 1997; Chen-Goodspeed et al. 2001; Cho et al. 2002; Yang et al. 2003). These studies have shown that the efficiency of the reaction can be increased by three orders of magnitude using 'difficult to cleave' substrates (Hill et al. 2003; Cho et al. 2004). Such variants may be necessary to improve yeast biocatalytic function against difficult to cleave organophosphate pesticides and nerve agents.

In conclusion, the results support the premise that yeast can function as a biosensor–biocatalyst for the detection and hydrolysis of paraoxon. Dual fluorescent biosensors with the ability to detect OP agents, catalyze their hydrolysis, and monitor the decontamination process will have potential in both the civilian and military sectors.

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