Appl Microbiol Biotechnol (2007) 74:1276–1283
DOI 10.1007/s00253-006-0758-y

Characterization of superoxide-stress sensing recombinant *Escherichia coli* constructed using promoters for genes *zwf* and *fpr* fused to *lux* operon

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Received: 11 January 2006 / Revised: 7 November 2006 / Accepted: 9 November 2006 / Published online: 11 January 2007

Abstract To measure the toxicity experienced by superoxide-generating compounds, two plasmids were constructed in which the superoxide-inducible *fpr* and *zwf* promoters from *Escherichia coli* were fused to promoterless *Vibrio fischeri luxCDABE* operon present in plasmid pUCD615. The bioluminescent response of *E. coli* harboring these constructs was studied as a function of the toxicity and was shown to be specific for superoxide generating chemicals. The two promoters employed, *fpr* and *zwf*, responded differentially to the redox-chemicals tested. Furthermore, a Δ*marA* strain bearing the *fpr*::*luxCDABE* fusion had a weaker response to paraquat (methyl viologen) than its isogenic parent strain, whereas *zwf* induction was not inhibited in Δ*marA* or Δ*rob* strains. The *fpr* and *zwf* promoters were also induced by alkylating agents but were unresponsive in Δ*marA* or Δ*rob* strains. Using optimized assay conditions, the abilities of these strains to differentially respond to superoxide stress and alkylating agents that may be present in contaminants proves them to be good biosensor candidates for monitoring toxicity.

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Introduction

Exposure of *Escherichia coli* to superoxide-generating agents, such as paraquat and related compounds, renders the cells resistant to higher doses of these agents. This protective action against the damage caused by oxidative stress is regulated by the SoxRS transcriptional factors. These factors induce the expression of antioxidant activities to the superoxide anion, $O_2^{\bullet-}$, through a number of inducible genes under the control of *soxRS* regulon (Greenberg and Demple 1989; Greenberg et al. 1990; Storz and Imlay 1999). The *soxRS* regulon positively modulates the expression of several genes, thereby mediating the oxidative stress response. The products of the induced *soxRS* regulon act collectively to reverse the effects of oxidative damage, including the re-reduction of oxidized metals in prosthetic groups by flavodoxin and ferredoxin reductase (*fpr*) and reconstitution of the NADPH pool by glucose-6-phosphate dehydrogenase (*zwf*) (Storz and Imlay 1999; Pomposiello and Demple 2001).

SoxRS-mediated *fpr* induction leads to increased levels of ferredoxin/flavodoxin-$NADP^+$ reductase (FPR) to maintain the reduced state of Fe–S clusters. Furthermore, *zwf* induction, as when cells are exposed to redox-cycling compounds such as paraquat (Fawcett and Wolf 1995; Kao and Hassan 1985; Krapp et al. 2002), leads to increased levels of glucose-6-phosphate dehydrogenase (G6PDH). The reduced ferredoxin and flavodoxin play an important role in the reductive activation of the number of enzymes of which are ribonuleotide reductase (Mulliez et al. 2001), methionine synthase (Fujii and Huennekens 1974), and pyruvate/formate lyase (Knappe et al. 1984). FPR plays a critical role in distributing low potential electrons that are largely derived from glucose metabolism. It appears to be that the reduction of SoxR could be caused by reduced

flavodoxin and ferredoxin and that the entire system is self-regulating because the gene coding for NADPH/ferredoxin reductase itself a member of *soxRS* regulon (Liochev and Fridovich 1992). In *E. coli*, expression of *zwf* is related to the cellular growth-rate and superoxide-generating agents (Rowley and Wolf 1991; Rowley et al. 1992). Both *fpr* and *zwf* are also members of the overlapping *marA/soxS/rob* regulon(s) (Jair et al. 1995). The promoters of genes included in the *marA/soxS/rob* regulon(s) have a marbox-binding site (Jair et al. 1995), which is composed of a 20-bp degenerate asymmetric recognition sequence where the ambidextrous MarA, SoxS, and Rob transcriptional factors bind and activate transcription (Li and Demple 1996; Jair et al. 1995; Fawcett and Wolf 1995; Martin et al. 2000). These genes confer multiple antibiotic resistance, superoxide resistance, and organic solvent tolerance (Martin and Rosner 2002; reviewed in Alekshun and Levy 1997). Despite overlapping regulation by SoxS, MarA, and Rob, these factors exhibit differential control on the individual genes. For instance, the Rob protein binds strongly to *E. coli* replication origin *oriC* and the *micF* gene but binds more weakly to the *sodA*, *nfo*, and *zwf* promoters (Ariza et al. 1995). Several promoters of *soxRS* regulon genes have identified marbox-binding sites, including *zwf, fpr, fumC, micF, nfo*, and *sodA* (Jair et al. 1995; Martin et al. 1999).

The measurement of toxicity, such as that caused by oxidative compounds, enables researchers to evaluate their harmful effects in biological systems. The most common method is to evaluate chemicals based upon the oxidative damage they cause to the cellular proteins, lipids, and certain prosthetic groups of some enzymes and DNA. The damage is then sensed through the translation of the signals into increased levels of antioxidant gene expression (Min et al. 1999; Van Dyk et al. 2001; Mitchell and Gu 2004). Promoters from the *soxRS* regulon genes have been fused to the *luxCDABE* genes in *E. coli* and have been applied in the monitoring of toxicity and in biosensor applications. These include a *micF*::*luxCDABE* fusion that was applied to wastewater monitoring (Belkin et al. 1996; Lee and Gu 2003), as well as to evaluate the effects of hypochlorous acid (Dukan et al. 1996) and cationic antimicrobial peptides (Oh et al. 2000). Furthermore, cellular arrays of several SoxRS regulon promoters in a random collection of *luxCDABE* fusions (Van Dyk et al. 2001) and a cell array biosensor (Lee et al. 2005) were also studied.

Recently, Kim et al. (2005) has proposed a methodology to search for promoter elements to develop whole cell-based biosensors through DNA microarrays. This study involves a similar approach by which the construction and characterization of two superoxide stress-responsive *E. coli* strains FPR/RFM443 and ZWF/RFM443 is described. These newly constructed strains harbor plasmids with the SoxRS-controlled *fpr* and *zwf* promoters for the ferredoxin/flavodoxin-$NADP^+$ reductase (*fpr*) and glucose-6-phosphate dehydrogenase (*zwf*) genes, respectively, fused to the *luxCDABE* genes from *Vibrio fischeri*. Using the plasmid-borne *fpr*::- and *zwf*::*luxCDABE* fusions, the bioluminescent responses that result when cells carrying these plasmids were exposed to various toxicants can be used as an indicator of promoter activity.

Materials and methods

Strains

All strains used in this study are listed in Table 1.

Chemicals

Paraquat [methyl viologen] (PQ), ethyl viologen (EV), benzyl viologen (BV), mitomycin C (MMC), and ethyl methanesulfonate (EMS) were purchased from Sigma (USA). Hydrogen peroxide [30% (wt/vol)] was obtained

Table 1 *E. coli* strains and plasmids used in this study

Strains/ plasmids	Relevant description	Reference
Plasmids		
pUCD615	Amp^r, Kan^r, *luxCDABE*, a promoterless plasmid	Rogowsky et al. (1987)
pZwfLux	pUCD615 with the *zwf* promoter fused to *luxCDABE*	This study
pFprLux	pUCD615 with the *fpr* promoter fused to *luxCDABE*	This study
Strains		
RFM443	*rpsL galK2, lacΔ74*	Menzel (1989)
FPR/ RFM443	As RFM443 but contains pFprLux	This study
ZWF/ RFM443	As RFM443 but contains pZwfLux	This study
AG100	*Gln V44* (AS), *galK2*(Oc), λ^-, *Δ(bioC-uvrB)*, *rpsL704*(strR), *xylA5, mtl-1, argE3*(Oc), *thi-1*	George and Levy (1983)
AG100kan	Same as AG100 but Δ(*marR-marB*)*876*(::kan)	Manneewannakul and Levy (1996)
RA4468	Δ(*lac*)*U169 rpsL*, Δ*rob*::kan	Ariza et al. (1995)
AGFPR1	As AG100 but contains pFprLux	This study
AGFPR2	As AG100kan but contains pFprLux	This study
AGZWF1	As AG100 but contains pZwfLux	This study
AGZWF2	As AG100kan but contains pZwfLux	This study
RAZWF	As RA4468 but contains pZwfLux	This study
RAFPR	As RA4468 but contains pFprLux	This study

from Merck (USA). All of the chemicals used in this study were of analytical grade.

DNA microarray analysis

One milliliter of 1 h chemical-treated and chemical-untreated cultures (*E. coli* RFM443) was used for DNA microarray analysis, and the methods for RNA extraction, labeling, reverse transcriptase PCR, hybridization, and processing of chips were same as previously described (Kim et al. 2005). The DNA microarray chips used in this study were the *E. coli* 6.0 K genome oligonucleotide microarray chips (Genomictree, Daejeon, Korea), and these were scanned with a Genepix 4000B laser scanner (Axon Instruments, CA, USA). The spot intensity analysis was performed with GenepixPro 3.0 software (Axon Instruments).

Construction of plasmids pZwfLux and pFprLux

Promoter regions upstream of the *zwf* or *fpr* genes were amplified by PCR using specially designed primers. The *zwf* promoter, which includes the binding sites for MarA and Rob (Martin and Rosner 2002) and corresponds to the −330-bp to +90-bp region with respect to the start codon of the *zwf* gene, was amplified from the *E. coli* RFM443 genomic DNA by PCR using the following primers 5′-ACT TAA **GGA TCC** GAT ATT ACG CCT GTG TGC CG-3′ and 5′-AGC AGC **GAA TTC** AGG GAA GGC AGC AAT TTA CG-3′. The resulting PCR product was digested with *Bam*HI and *Eco*RI, the sites for which are shown in the primers as bold type and ligated into pUCD615 using the same sites (Rogowsky et al. 1987) to yield plasmid pZwfLux, in which the *luxCDABE* genes from *V. fischeri* were controlled by the *zwf* promoter. The *fpr* promoter region, from −203 bp to +94 bp with respect to the start codon and includes the MarA binding site, was amplified with the following primers: 5′-ACT TAA **GGA TCC** TAC CGA AAC GCT GCT GAT CC-3′ and 5′-AGC AGC **GAA TTC** TAA ACG GAA GCA CGG GGG CG-3′. The same procedure as above for *zwf* was used to create plasmid pFprLux.

The plasmids were transformed into *E. coli* RFM443, and positive colonies were selected on Luria–Bertani (LB) agar supplemented with ampicillin (50 μg/ml) and kanamycin (25 μg/ml). The bioluminescent transformants carrying the recombinant plasmids (pZwfLux or pFprLux) were confirmed by restriction digestion, PCR with the respective primers, and sequence analysis of the amplified products.

Experimental conditions

An *E. coli* host strain, RFM443 (*rpsL*-(StrR), *galK2*, *lac*Δ*74*) was transformed with the newly constructed plasmids pZwfLux and pFprLux, giving rise to strains ZWF/RFM443 and FPR/RFM443, respectively. These plasmids were also transformed in to other *E. coli* strains for further characterization (Table 1). All recombinant strains were grown in Luria–Bertani (LB) medium (DIFCO, USA), which included ampicillin (50 μg/ml) and kanamycin (25 μg/ml) to maintain the plasmids.

Preparation of the cells and bioluminescence measurement

Experiments were setup as described previously by Mitchell and Gu (2004) with slight modification when needed. Briefly, the strains were initially grown overnight in 2-ml LB medium with ampicillin and kanamycin in 10-ml Falcon tubes in a shaking incubator set at 300 rpm and 37°C. The overnight culture was diluted 100-fold into fresh LB medium supplemented with ampicillin (50 μg/ml) and incubated at 37°C in shaking incubator at 300 rpm. For bioluminescence measurements, the cells were grown until the optical density at 600-nm reached was 0.08, after which 100 μl of this culture was mixed with an equal volume of LB containing the test chemical in an opaque 96-well plate (Microplate 1, Dynex, USA), giving a final volume of 200 μl. Each of the test compounds were diluted into LB medium just before the addition of the culture.

Experiments were performed by incubating the 96-well plate in a plate luminometer (Microtitre Plate Reader, MLX, USA) at 30°C that was set to shake the plate for 10 s before each reading. The bioluminescence levels were recorded every 10 min. Results are presented as the relative bioluminescence (RBL), which is defined as the ratio of bioluminescence (arbitrary light units) of chemically-treated sample to that of untreated sample and plotted against concentration of the test chemical. The error bars show the standard deviations, which were calculated using a minimum of three independent experiments.

Results

Construction of the *zwf*::- and *fpr*::*luxCDABE* gene fusion harboring strains ZWF/RFM443 and FPR/RFM443

Preliminary genome-wide transcription profiles of *E. coli* cells exposed to superoxide-generating compounds were determined using DNA microarray analysis. The cells growing in LB broth were exposed to 0.05 mM of PQ [methyl viologen], EV, or BV for 1 h. The microarray results compared the expression profiles of treated with untreated cultures and genes that were strongly induced by PQ, EV, and BV are listed in Table 2. Interestingly, many of the genes listed are not included in the SoxRS-regulon. However, eight of these genes, listed in Table 2, are

commonly induced by all three viologens (PQ, EV, and BV), seven of which are members of the SoxRS-regulon. As the *fpr* gene showed the highest response, whereas *zwf* gave the lowest, these two genes were selected as candidates for further characterization and to develop oxidative-stress-sensing cells. The new *E. coli* strains, i.e., FPR/RFM443 and ZWF/RFM443 and harboring pFprLux and pZwfLux, respectively (Table 1) were constructed by fusing the promoters of the *fpr* and *zwf* genes with the *V. fischeri luxCDABE* genes within plasmid pUCD615.

Responses of FPR/RFM443 and ZWF/RFM443 to oxidative agents

FPR/RFM443 and ZWF/RFM443 were characterized by their bioluminescent responses when exposed to PQ, a superoxide-generating reagent. Dose-dependent bioluminescent responses were seen from both strains when exposed to various concentrations of PQ, whereas the maximum induction was achieved within 180–230 min after initiating the exposure (Fig. 1a). FPR/RFM443 was strongly induced by PQ up to a concentration of 77.8 mM, above which its response decreased, presumably due to the overt toxicity of the sample (Fig. 1a). On the other hand, ZWF/RFM443 showed a maximum response at only 9.7 mM PQ (Fig. 1a). Induction of both promoter fusions began to decline 180–230 min after the exposure began. This loss in bioluminescence is likely due to a lower metabolic activity of the cells as they entered the stationary

Table 2 Response ratio's seen from the most strongly induced *E. coli* genes after treatment with 0.05 mM of PQ, EV, or BV for 1 h

Gene[a]	Function	Paraquat	Ethyl viologen	Benzyl viologen
fpr	Ferredoxin-NADP reductase	126.4	54	70.3
soxS	Regulation of superoxide response regulon	42.8	30.6	82.4
ydbK	Putative pyruvate-flavodoxin oxidoreductase, Fe–S subunit	57.9	15.9	36.7
fumC	Fumarase C=fumarate hydratase Class II	26.8	16.1	28.2
yjbK	Putative regulator	17.6	11	14.3
nfo	Endonuclease IV	16.6	11.4	25.1
ribA	GTP cyclohydrolase II	15.6	14.2	17.1
zwf	Glucose-6-phosphate dehydrogenase	7.4	5.6	10.1

[a] Genes, which are commonly induced by PQ, EV, and BV with an expression ratio at least five are shown in the table

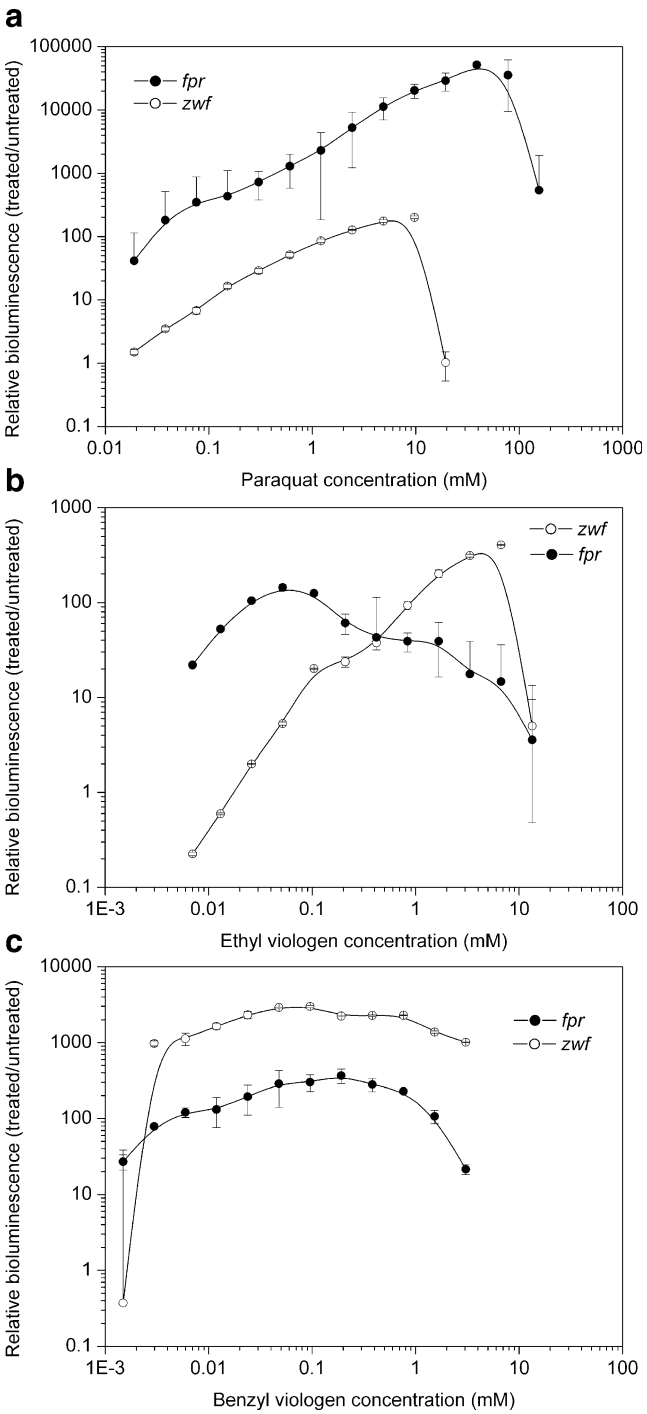

Fig. 1 **a** RBL responses of FPR/RFM443 (*fpr*::*luxCDABE*) and ZWF/RFM443 (*zwf*::*luxCDABE*) to various concentrations of PQ. RBL values were calculated as bioluminescence (arbitrary light units) of treated sample divided by that of the untreated sample. **b** RBL responses of FPR/RFM443 (*fpr*::*luxCDABE*) and ZWF/RFM443 (*zwf*::*luxCDABE*) to various concentrations of EV. RBL values were calculated as bioluminescence (arbitrary light units) of sample divided by that of the untreated sample. **c** RBL responses of FPR/RFM443 (*fpr*::*luxCDABE*) and ZWF/RFM443 (*zwf*::*luxCDABE*) to various concentrations of BV. RBL values were calculated as bioluminescence (arbitrary light units) of sample divided by that of the untreated sample

phase of growth or a limitation in oxygen brought on by high cell numbers (Meighen 1991; Neilson et al. 1999).

The responses of these strains to analogs of paraquat were tested next. The results showed that the strain ZWF/RFM443 was highly induced by EV and was more responsive to elevated concentrations of EV when compared to FPR/RFM443 (Fig. 1b). However, the response pattern from strain FPR/RFM443 (*fpr*) differed greatly from its response to PQ, with a maximum response of 144-fold seen with only 0.042 mM EV (Fig. 1b). For concentrations greater than this, the response ratio was much lower but stable at about 40. Likewise, ZWF/RFM443 (*zwf*) showed a much stronger dose-dependent response to BV than FPR/RFM443 (*fpr*). The concentration giving the maximum response with FPR/RFM443 was 0.143 and 0.095 mM for ZWF/RFM443. The strains were still responsive at higher concentrations, but the responses declined in a dose-dependent fashion (Fig. 1c). Based upon their responses to each of these compounds and the range of responsive concentrations, *fpr* was strongly induced by PQ, whereas *zwf* was more responsive to EV and BV.

To test whether H_2O_2 (a different oxidative damaging agent) could induce the *fpr* or *zwf* genes, fresh cultures were exposed to a range of hydrogen peroxide concentrations (0.005 to 20 mM). The relative bioluminescent responses with 0.35, 0.75, 1.5, and 20 mM hydrogen peroxide were 0.85, 1.9, 2.0, and 1.45, respectively, with *fpr*::*luxCDABE* fusion and 1.82, 1.94, 1.7, and 0.58, respectively, with the *zwf*::*luxCDABE* fusion. None of the concentrations tested led to an induction of greater than 2.0 during the 230-min exposure. These results demonstrate that the *fpr* and *zwf* genes are not substantially induced by hydrogen peroxide.

Induction of *zwf* and *fpr* by superoxides in Δ*marA* or Δ*rob* strains

It is documented that induction of these genes is controlled by *marA* and/or *rob* along with *soxRS* (Martin et al. 1999). Therefore, it would seem reasonable that the deletion of the *marA* or the *rob* gene would alter the responses of the *fpr* or *zwf* fusions when they experience superoxide stress. To test this, *E. coli* strains carrying either a *marA* or *rob* deletion mutation were transformed with the plasmids carrying the *fpr*::*luxCDABE* or *zwf*::*luxCDABE* fusions. The transformants were then characterized by exposing them with a wide range of paraquat concentrations. It was found that *zwf* was induced by PQ in both the wild-type strain (AGZWF1) and its *marA* isogenic mutant (AGZWF2; Fig. 2). Strain AGZWF1, in which *marA* was intact, displayed slightly higher induction levels than AGZWF2, suggesting that *marA* weakly contributed to *zwf* gene induction by superoxide stress (Fig. 2). On the other hand, the *fpr* induction by PQ was significantly lower in the *marA*-deleted strain (AGFPR2) compared to its isogenic parent strain (AGFPR1) in which *marA* is intact (Fig. 2). These results strongly suggest that the *zwf* gene is induced in the absence of *marA*, whereas *fpr* essentially required *marA* for its induction by superoxides.

Induction of *zwf* by superoxides was also conducted in a *rob* deletion mutant strain (RAZWF) to see the effect this gene has on *zwf* expression, as it was shown previously to be regulated by *rob* (Ariza et al. 1995). Our results clearly show that the absence of the *rob* gene had no effect on the induction of *zwf*, which was induced several thousand-fold by PQ relative to the untreated cells (RAZWF) (Fig. 3).

Response of FPR/RFM44 and ZWF/RFM44 to other toxicants

The responses of FPR/RFM443 and ZWF/RFM443 to genotoxins were also tested. Both strains were only weakly responsive to MMC when compared to the redox compounds. However, FPR/RFM443 was both much more sensitive and more responsive to MMC (RBL=130 at 7.4 μM). On the other hand, ZWF/RFM443 showed a stable RBL with the higher MMC concentrations, whereas that of FPR/RFM443 dropped (Fig. 4a). Additionally, both strains were also stimulated by EMS, a known alkylating agent, and the results were similar as those seen with MMC (Fig. 4b). These results indicate that these compounds might generate oxidative stress in the cells but only weakly so, as shown by the inductions seen with redox compounds. This suggests that the *soxRS* regulon is not the only regulator of *fpr* and *zwf*, particularly under alkylating conditions but that they could also be under the control of other genes, such as *rob* or

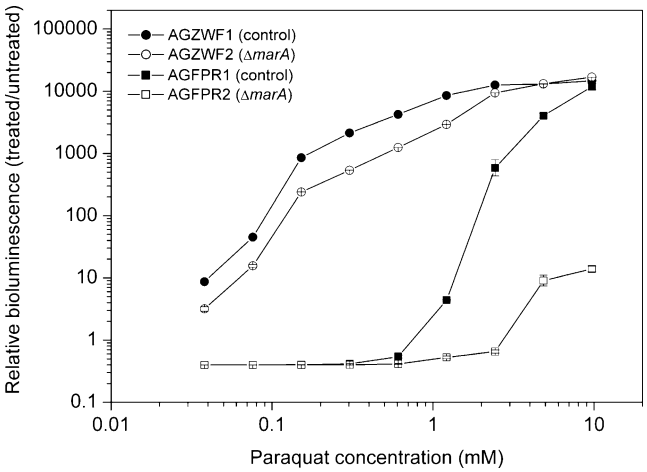

Fig. 2 Induction profiles of *marA* deletion mutant strains AGFPR2 (*fpr*::*luxCDABE*) and AGZWF2 (*zwf*::*luxCDABE*) compared to their isogenic parent strains AGFPR1(*fpr*::*luxCDABE*) and AGZWF1 (*zwf*::*luxCDABE*) after being treated with various concentrations of PQ

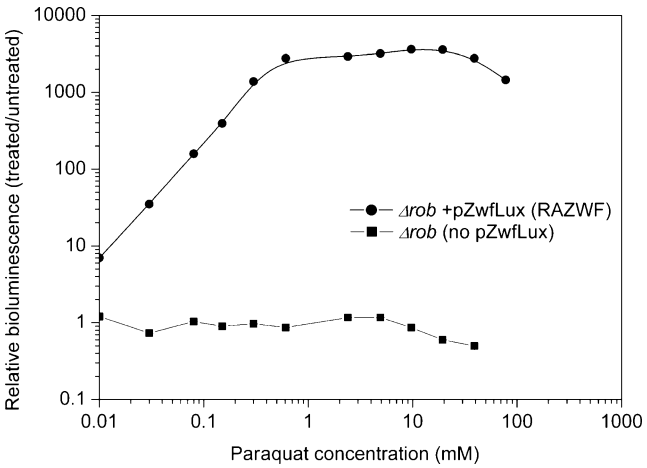

Fig. 3 Response of the *rob* deletion mutant strain RAZWF (*zwf*::*luxCDABE*) and strain RA4468 after exposure to various concentrations of PQ. The values are the RBL (arbitrary light units) of the PQ-treated sample divided by that of the untreated sample

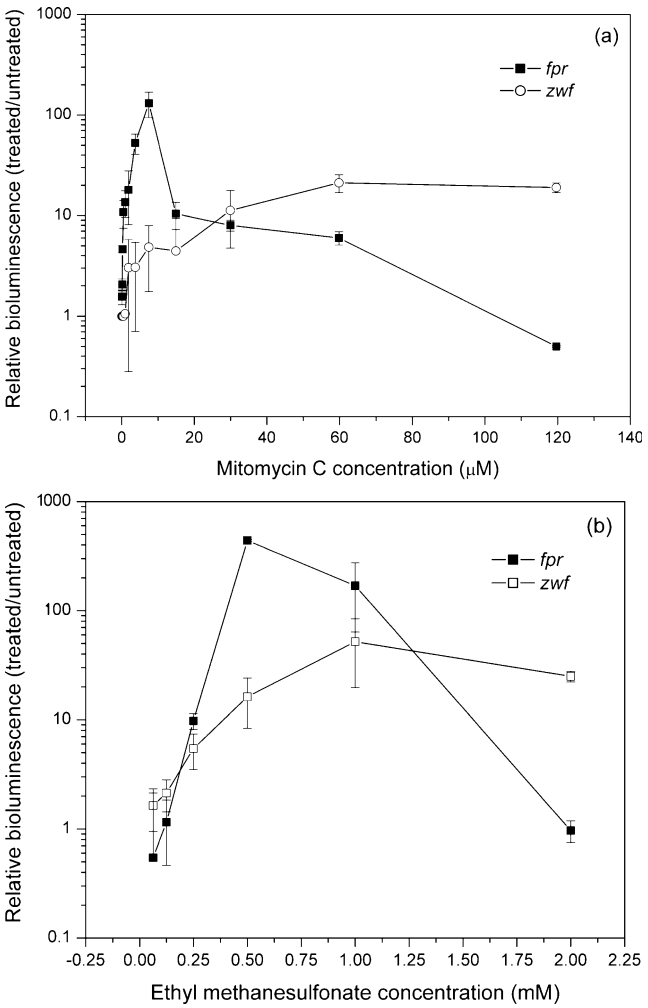

Fig. 4 Induction of *fpr* (FPR/RFM443) and *zwf* (ZWF/RFM443) when treated with alkylating agents: **a** mitomycin C and **b** ethyl methanesulfonate. RBL values were calculated as bioluminescence (arbitrary light units) of sample divided by that of the untreated sample

marA (Li and Demple 1996; Jair et al. 1995; Fawcett and Wolf 1995; Martin et al. 2000). To test the hypothesis that the induction of *fpr* and *zwf* by alkylating agents is mediated by the *marA* or *rob* gene, AGZWF2, AGFPR2, RAZWF, and RAFPR were treated with varying concentrations of the above two alkylating agents. None of these strains showed any significant responses (data not shown). As RFM443 bearing both promoter-*lux* fusions showed very significant induction levels (Fig. 4a,b), these results strongly suggest that the responses of FPR/RFM443 and ZWF/RFM443 to alkylating agents occur through the involvement of the *marA* or *rob* genes.

Discussion

Screening of *E. coli* cells exposed to PQ, EV, and BV by DNA microarray analysis found numerous genes that were strongly induced (Table 2) and were consistent with a previous report (Pomposiello and Demple 2001). We found that the *E. coli* genes, which are induced by chemical analogs of paraquat varied depending on the induced gene type and their response ratios. Of these genes, only a few were commonly induced by each of the test compounds, whereas all but one of these genes are known to be members of the SoxRS regulon. The *fpr* gene was the most strongly induced gene, whereas *zwf* showed the weakest induction but still had an induction ratio of greater than 5. To characterize and compare the responses of the *fpr* promoter with the other genes, we selected the *zwf* promoter, as it showed the greatest difference based upon DNA microarray analysis.

The *E. coli fpr* and *zwf* promoters were stimulated by oxidative damage resulting from exposure to redox-cycling agents, such as viologens, and reached a maximum induction within 3 to 4 h of initiating the exposure. The *fpr* and *zwf* promoters differed in their responses to the three different viologens (PQ, EV, and BV), all of which are known to cause superoxide stress. These viologens differ in their structures, with both PQ and EV having different alkyl groups, whereas BV has aromatic groups. Due to these variations in their structures, these viologens likely have different redox-cycling activities which, leading to the differences seen in the *fpr* and *zwf* responses. For instance, *fpr* responded the strongest to PQ, but *zwf* was highly induced by exposure to EV and BV. Using the differential responses of *fpr* and *zwf* to these different viologens, it should be possible to rank such chemicals according to their redox-cycling activity or toxicity.

Expression of *fpr* provides increased PQ tolerance to *E. coli* cells (Bianchi et al. 1995; Krapp and Carrillo 1995), and

these results are consistent to the PQ tolerance of FPR/RFM443 (*fpr*) to higher doses (Fig. 1a). The protective effects of the *fpr* gene product, i.e., the ferredoxin (flavodoxin)-NADP(H) reductase (FPR), are linked to its NADP(H)-dependent activities. FPR induction by the *soxRS* response could be involved in maintaining the cellular NADPH concentrations at tolerable levels. The reduction of ferredoxin and flavodoxin by FPR plays an important role in the reductive activation of number of enzymes (Liochev et al. 1994; Arakaki et al. 1997; Fridovich 1997; Krapp et al. 2002; Li and Demple 1996). Similarly, the *zwf* gene encodes for the G6PDH, which is also regulated by *soxRS*, and maintains a high NADPH/$NADP^+$ ratio and thus, provides a reducing environment (Liochev and Fridovich 1992; Storz and Imlay 1999). Strain ZWF/RFM443 was also responsive to higher concentrations of both EV and BV, but it is unclear why this is true. However, both of these compounds have larger side groups than paraquat, which can alter their reactivity. Interestingly, a comparison of both the *fpr* and *zwf* responses to paraquat with previously reported biosensors bearing a *soxS–lux* fusion found that the *fpr* promoter was induced more strongly than *soxS* promoter, i.e., the RBL was higher (Kim et al. 2005). In contrast, the responses from the *zwf* promoter were weaker than the *soxS–lux* fusion strain, which is what was expected.

To test whether *fpr* or *zwf* could be induced by higher levels of H_2O_2, the cells were treated to a wide range of H_2O_2 levels. No relative bioluminescent responses greater than twofold were seen. Because only values showing at least a 2.5-fold induction within 230 min after exposure were deemed significant, hydrogen peroxide had no clear effect on the expression of these genes. The fact that these *E. coli* strains constructed can distinguish specific target chemicals by their distinct responses demonstrates that they can be used as specific biosensors for the detection and classification of toxicants.

In addition, the fact that alkylating agents induce *fpr* and *zwf* indicates that these genes are controlled not only by *soxRS* but also by other genes. It is well documented that both *fpr* and *zwf* are also regulated by *marA* and that the *zwf* gene is also regulated by *rob* (Jair et al. 1995; Ariza et al. 1995; Martin and Rosner 2002). These overlapping regulatory systems, which include the *marA*/*soxS*/*rob* regulon(s), share an asymmetric 20-bp recognition sequences called the marbox, which is positioned within the promoter regions of the *marA*/*soxS*/*rob* regulon genes (Li and Demple 1996; Jair et al. 1995; Fawcett and Wolf 1995; Martin et al. 2000). We therefore examined the effect of *marA* or *rob* deletions on the induction of *fpr* or *zwf* when exposed to viologens and alkylating agents. In *rob* mutants, there was no obvious difference in the *zwf* expression when the culture was exposed to superoxides, which is consistent with a previous study that found Rob had no effect on *zwf* induction in vivo (Ariza et al. 1995). Furthermore, the responses of these two genes to alkylating agents were lost. This suggests that the induction of *fpr* or *zwf* by alkylating agents may be mediated either by the MarA or Rob proteins or via the activation of the SoxRS response through an unknown pathway.

Acknowledgement This work was supported by the Korea–Israeli Joint Fund Program of Ministry of Science and Technology (MOST) and in part by KOSEF through the Advanced Environmental Monitoring Research Center (ADEMRC) at the Gwangju Institute of Science and Technology (GIST). The authors express their gratitude for this support.

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