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Construction of a Reporter Strain *Pseudomonas putida* for the Detection of Oxidative Stress Caused by Environmental Pollutants

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Abstract A green fluorescent protein-based *Pseudomonas putida* reporter was successfully constructed and shown to be capable of detecting oxidative stress. In this whole-cell reporter, the promoter of the paraquat-inducible ferredoxin-$NADP^+$ reductase (*fpr*) was fused to a promoterless *gfp* gene on a broad-host-range promoter probe vector. *Pseudomonas putida* KT2440 harboring this reporter plasmid exhibited an increased level of *gfp* expression in the presence of redox-cycling agents (paraquat and menadione), hydrogen peroxide, and potential environmental pollutant chemicals such as toluene, paint thinner, gasoline, and diesel. Induction of *fpr* in the presence of these chemicals was confirmed using Northern blot analysis.

Key words: Paraquat, menadione, paint thinner, hydrogen peroxide, green fluorescence protein

The reactive oxygen species (ROS), such as superoxide and hydroxyl radicals, are formed routinely during a variety of biochemical reactions and cellular functions [2, 25]. These ROS must intracellularly be managed by antioxidant chemicals or enzymes that allow bacterial cells to cope with ROS toxicity [6]. Bacteria have evolved adaptive defense systems to protect themselves against the detrimental effects of these ROS [6]. Cells can usually tolerate mild oxidative stress, which induces antioxidant defense enzymes by the coordinate regulation of specific genes [6, 19]. Such adaptive stress regulons in *Escherichia coli* have been extensively studied [11, 21]. The SoxRS regulon plays important roles in protecting *E. coli* cells from superoxide and nitric oxide stresses [21]. OxyR responds to cellular exposure to hydrogen peroxide (H_2O_2) by stimulating transcription of many oxidative stress defense proteins (e.g., katG, ahpC, gorA, dps, etc) [27]. However, identification of redox-regulated transcriptional factors and their role in soil microorganisms have been poorly characterized.

Pseudomonas putida KT2440 is a metabolically versatile, saprophytic soil bacterium that has been extensively studied as an experimental model soil microorganism [13, 16]. Previously, the *fpr* gene (encoding the ferredoxin:$NADP^+$ reductase) of *Pseudomonas putida* KT2440 has been shown to be induced in the presence of redox-cycling reagents such as the herbicide methyl viologen (paraquat) (Park and Demple, unpublished data). In *E. coli*, disruption of the *fpr* increases sensitivity to paraquat [4]. The expressed gene product, FPR, can mediate reversible oxidation of NADPH by a wide variety of electron acceptors such as viologens, quinones, and complexed transition metals [6, 15]. Thus, FPR may generally be involved in the protection of the bacteria from damage by oxidative stress. This notion is supported by the fact that the *fpr* gene in *E. coli* belongs to the SoxRS regulon [15]. Here, we fused the promoter of the paraquat-inducible *fpr* gene to a promoterless *gfp* gene (encoding green fluorescence protein), creating a reporter strain sensing oxidative stress caused by a variety of environmental chemicals. GFP has been widely used to construct various biosensors because of its stability and utility in the absence of added substrates [10, 12]. Recently, several attempts to develop biosensors for the detection of presence of specific environmental pollutants have been reported [3, 17]. In the present study, we developed a reporter strain from a model soil microorganism, *P. putida* KT2440, and found that the *fpr-gfp* fusion has potential for detecting oxidative stress caused by environmental pollutants.

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MATERIALS AND METHODS

Bacterial Strains, Culture Conditions, and DNA Manipulation

Bacteria were grown at 37°C (*E. coli*) or 30°C (*Pseudomonas*) in Luria-Bertani (LB) medium with vigorous aeration by shaking at 220 rpm. All chemicals including paraquat, menadione, and H_2O_2 were added at the final concentrations described in the figure legends. Plasmid isolation, gel electrophoresis, transformation, and polymerase chain reaction [(PCR) DNA amplification] were performed by standard procedures [1]. *P. putida* KT2440 (Laboratory stock) was used for constructing the reporter strain. *P. putida* KT2440-R was selected from spontaneous mutations on LB medium containing rifampin (200 mg/ml), after prolonged incubation and sequential transfers. *E. coli* S17-1 was used as the donor for conjugative transfer of plasmids [23]. Tetracycline (15 μg/ml) and rifampin (200 μg/ml) were added to bacterial cultures when necessary.

Construction of the *fpr-gfp* Fusion in *P. putida* KT2440

A broad-host-range promoter probe vector, pRK415gfp [26], was obtained from Dr. James Shapleigh (Cornell University, U.S.A.) and used for constructing a reporter plasmid, pFPRgfp. Briefly, a 417-bp fragment from the *fpr* promoter region was amplified using primers Fpr-pro1 (CGCGAATTC-GCCGTTGAGCAGGTCTTC) and, Fpr pro3 (CGCGGATCC-ACGTTCGTGGTTCATGTTGCTCA). The amplicon was cloned into the EcoRI/BamH1 cloning sites of the pRK415gfp vector, generating pFPRgfp. The constructed plasmid was introduced into *E. coli* S17-1 by electroporation. Both directions of the inserted fragments were sequenced, and all sequencing was completed at Bionex (Seoul, Korea). Then, the pFPRgfp plasmid was introduced by biparental conjugation into *P. putida* KT2440-R, thus creating *P. putida* KT2440 (pFPRgfp). Conjugation was performed by filter mating with *E. coli* S17-1 (pFPRgfp) and *P. putida* KT2440-R as donor and recipient, respectively [20].

Microscopic Analysis

Bacterial cells with the *fpr-gfp* fusion were viewed with an Axioskop 2 epifluorescent microscope with low-power objectives for scanning and a 100× Plan-Neofluar Objective, N.A 1.30 for epifluorescent imaging and phase contract microscopy. The microscope is equipped with a fluorescence filter cube for detecting GFP (09 GFP, Carl Zeiss). Images were obtained with an Axiocam. The AxioVision software (AxioVision Version 3.1) was used to acquire images. For each image, the background intensity was measured with an area lacking cells.

Quantification of GFP Fluorescence

Exponentially grown bacterial cells (OD_{600}=~0.3) in 50-ml flasks were treated with a variety of environmental chemicals. The concentration of each chemical is shown in the figure legends. After exposure to each chemical for 1 to 24 h, 2 ml of cells suspension was collected using a microcentrifuge (~13,000 rpm) and washed twice with 800 ml of phosphate-buffered saline (PBS). Then, both OD_{600} of resuspended cultures and GFP fluorescence intensity were quantified using a microtiter plate reader (VICTOR^3, Biorad). The reporter strain expresses a stable GFP variant that absorbs at 488 nm.

Northern Blot Analysis

Total RNA was isolated from 3 ml of exponentially growing cells using a RNeasy kit (Qiagen), following the manufacturer's instructions. RNA concentration was measured by the absorbance at 260 nm. Ten μg of total RNA per sample was used. The fractionated RNA was transferred to a nylon membrane (Schleicher & Schuell) with a Turboblotter (Schleicher & Schuell). The amount of the *fpr* mRNA was determined by hybridizing the membrane with a *fpr*-specific ^{32}P-labeled probe (Invitrogen) [1] prepared by PCR amplification with primer pair, fpr Pp-1 (CCATCGCTTC-GCCAAACTG)/fpr Pp-2 (GCCGGTGCTCAGCAGGTAC).

RESULTS AND DISCUSSION

Construction of Reporter Strains Responding to Paraquat

Previous work demonstrated that the promoter of the *fpr* gene of *P. putida* KT2440 has shown to be strongly induced in the presence of the superoxide-generating reagent, paraquat, and that this induction requires a LysR-type transcriptional factor divergently transcribed from the *fpr* promoter (Park and Demple, unpublished data). By inserting a 417-bp fragment from the *fpr* promoter region into cloning sites of pRK415gfp, the superoxide-sensing reporter plasmid pFPRgfp was constructed. This reporter plasmid was introduced into the soil model bacterium *P. putida* KT2440 to measure oxidative stress caused by paraquat. Bacterial cultures were freshly grown on LB agar plates in the absence and presence of paraquat (0.2 mM). After overnight growth of the reporter strain, bacterial cells were scraped with an inoculation loop and placed on a glass slide for microscopic analysis. As shown in Fig. 1A (panel d), strains containing pFPRgfp produced a high level of GFP in the presence of paraquat, whereas this strain produced significantly less amount of GFP in the absence of paraquat (Fig. 1A, panel b). The amount of expressed GFP, quantified with a microtiter plate reader, indicated that GPF expression with paraquat (0.2 mM) was 2- to 3-fold higher than without paraquat (Fig. 1B). These results suggested that GFP expression in the reporter strain reliably reports oxidative stress.

Concentration- and Time-Dependent GFP Inductions

The GFP expression, measured by fluorescence intensity in LB liquid culture, was examined at 4 paraquat concentrations

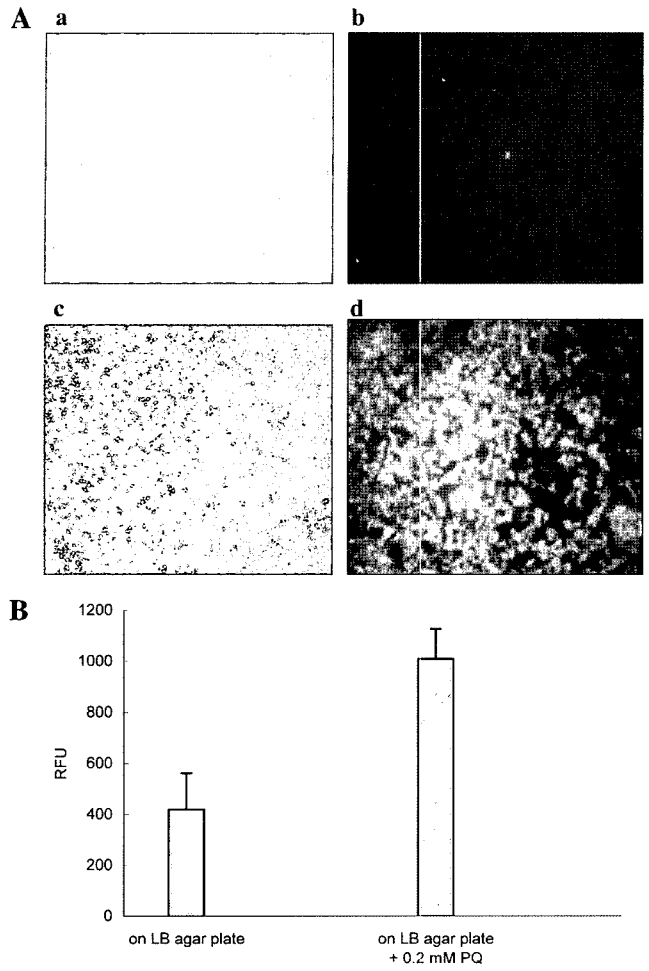

Fig. 1. A. Microscopic analysis of *P. putida* KT2440 (pFPRgfp) reporter strain responding to oxidative stress (a, b: phase contrast, epifluorescent observations of the reporter strain in the absence of paraquat, respectively; c, d; phase contrast, epifluorescent-observation of the reporter strain in the presence of paraquat, respectively. **B.** Quantification of GFP expression in the reporter strain in the absence and presence of paraquat (0.2 mM). Bacterial cells were collected from overnight culture grown in LB agar plate. RFU (relative fluorescence unit) is defined as culture fluorescence divided by culture OD_{600}.
The cultures were washed with PBS buffer twice before fluorescence measurement. Mean values for three independent experiments are shown with standard deviation.

over 4 exposure periods (Fig. 2). Response generally increased with increasing paraquat concentration; however, inhibition was observed at the highest concentration of paraquat. High concentrations of paraquat are toxic and are likely to severely inhibit bacterial metabolism [8]. This means that quantification is not possible using this reporter strain at high concentration of pollutant/toxic compound. We observed that the highest concentration of paraquat stopped bacterial growth or caused death (data not shown). The absence of a linear response of the reporter strain to paraquat concentration may also reflect limited synthesis of regulator protein and/

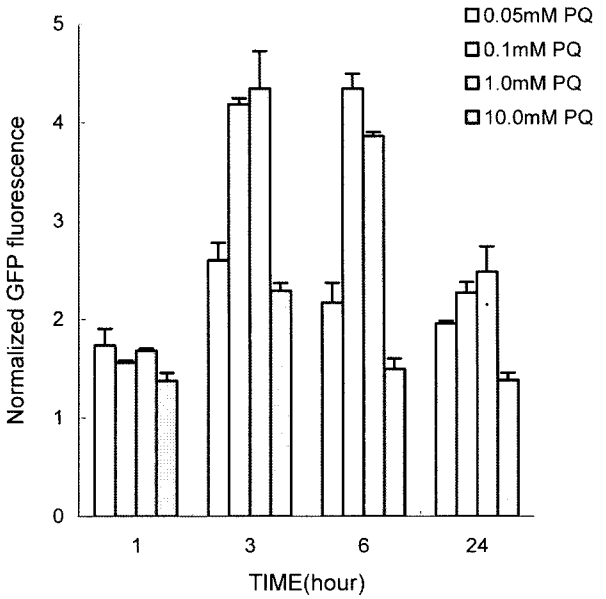

Fig. 2. Oxidative stress response of *P. putida* KT2440 (pFPRgfp) liquid culture to various concentrations of paraquat for four exposure periods.
Normalized GFP fluorescence was defined as: [(fluorescence intensity/OD_{600} of exposed cells)/(fluorescence intensity/OD_{600} in chemical-untreated bacterial cells)]. Mean values for three independent experiments are shown with standard deviation.

or saturation of promoter binding sites [7]. Regarding exposure times, the GFP response showed a maximum at 3–6 h (Fig. 2). The decrease of the normalized GFP fluorescence after 24 h was observed (Fig. 2). This reduction may be due to decrease of GFP production, whereas bacteria growth continues.

Response of the Reporter Strain to Various Environmental Chemicals

The reporter strain was used to detect oxidative stress generated by various environmental pollutants. The reporter strain freshly grown in LB liquid culture (OD_{600} ~0.3) was incubated in the absence or presence of each pollutant for an additional three hours with the concentration shown in Fig. 3. Bacterial culture in LB liquid medium was used as a control against which all other treatments were compared. Addition of succinate (10 mM) and glucose (2%) as non-toxic chemicals did not increase GFP expression of the reporter strain (Fig. 3). Redox-cycling reagents (paraquat and menadione) and hydrogen peroxide increased the *fpr-gfp* expression significantly. To our best knowledge, this is the first report indicating that the *fpr* expression of *Pseudomonas* strain is highly inducible with hydrogen peroxide. Consistent with this observation, Zheng *et al.* [28] has shown using microarray studies that the *fpr* of *E. coli* is one of the strongly induced genes with hydrogen peroxide.

Among the environmental chemicals tested here, paint thinner (1%) and stain cleaner (0.5%) increased the GFP expression the most. Paint thinner and stain cleaner are

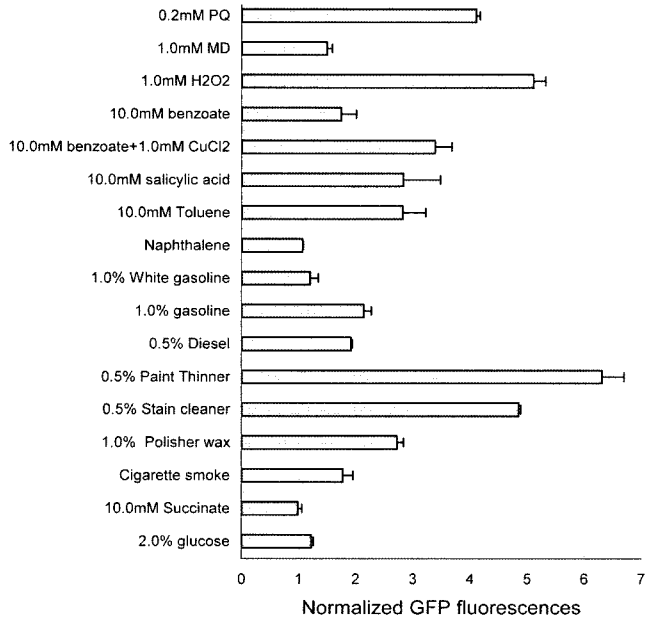

Fig. 3. Effect of potential environmental pollutant chemicals on the *fpr-gfp* expression in *P. putida* KT2440. The reporter strain was exposed to each chemical for three hours.
Mean values for three independent experiments are shown with standard deviation. For cigarette smoke effect, two puffs of smoke from a commercially available cigarette was exhaled into a 15-ml Falcon tube and the lid was closed, and then incubated at 30°C with shaking for three hours. Normalized GFP fluorescence was determined as described in the legend of Fig. 2. Background control treatments were bacterial culture in LB without any pollutant. Background control measurement was done after the same additional three hours of incubation without pollutant.

probably turpentine substitutes that are hydrotreated light distillates of petroleum. It is virtually certain that these preparations contain more than one oxidative stress-causing chemical. Other petroleum products such as gasoline, diesel, or wax polisher also increased the expression of *gfp*, as shownin Fig. 3. All peroleum products tested induced the GFP expression in a concentration-dependent manner (data not shown). Interestingly, white gasoline (0.1%–1%) with different incubation time (1–6 h) did not change the GFP expression compared with no treatment. White gasoline is a more refined distillated gasoline without additives, and it is probably less toxic to bacterial cells within those concentration ranges. In the presence of heavy metals (e.g., copper, iron), many organic pollutants can generate the ROS inside bacterial cells [22]. This is consistent with our present observation that the GFP expression was slightly higher with addition of benzoate and Cu^{2+} together, compared with the GFP expression by benzoate addition alone (Fig. 3). Up to 3- to 4-fold induction occurred with toluene or salicylate (the latter is a common environmental chemical found in soil or the rhizosphere). Napthalene (30 ppm), not a substrate for metabolism by *P. putida* KT2440, did not induce GFP expression above background levels. Although it is highly possible that, when environmental chemicals have a low

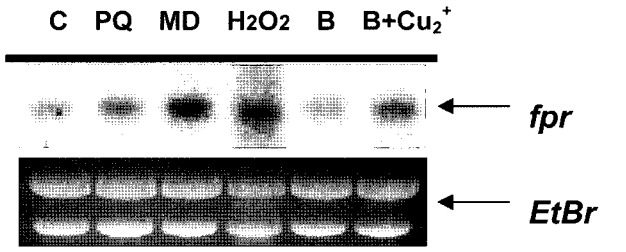

Fig. 4. Induction of the *fpr* gene by paraquat (PQ, 0.2 mM), menadione (MD, 0.5 mM), hydrogen peroxide (H_2O_2, 5 mM), and benzoate (B, 10 mM). Cu^{2+} (0.1 mM) was added when necessary. C: control culture without any additional chemical.
Total mRNA was extracted at 10 min exposure after treatment of cells by the above chemicals. The ethidium bromide-stained (EtBr) gel prior to blotting is also shown in Northern blot analysis to demonstrate consistent loading in all lanes.

solubility in water, it might not be accesible to bacterial cells, and naphthalene clearly did not create oxidative stress in non-naphthalene-degrading bacteria at the exposure concentration.

fpr mRNA Quantification by Northern Blot Analysis

Northern blot analysis was conducted to confirm the expression of the *fpr* gene in the presence of several environmental chemicals. Consistent with the *fpr-gfp* expression patterns obtained with the reporter strain, Northen blot data confirmed that the *fpr* induction was much higher in the presence of benzoate in combination with Cu^{2+} (Fig. 4). Hydrogen peroxide and superoxide-generating reagents induced the transcription of the *fpr* gene (Fig. 4). Our findings indicate that this GFP-based reporter strain offers promise as a useful tool for detecting oxidative stress caused by environmental chemicals, although the response of this reporter to environmental chemicals is not sensitive. It has been shown that the GFP reporter is not sensitive, compared with constructs using the *lux* reporter [9]. Additional improvement and refinements, especially regarding the toxicity of test chemicals, should confirm and extend the relationships between this GFP expression system and chemical-mediated cellular injury. For soil microorganisms, oxidative stress can be caused by exposure to environmental pollutants, as well as by the soil fungus *Talaromyces flavus* [5, 14, 18]. Oxidative stress caused by metabolism of many pollutants can result in cellular damage [5, 16, 24] and has important implications for managing soil microorganisms that facilitate bioremediation processes. Further studies using a modified reporter strain will provides a new framework for the elucidation of the physiological basis of the metabolic versatility and environmental stress response of soil microorganisms.

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