Overexpressing antioxidant enzymes enhances naphthalene biodegradation in *Pseudomonas* sp. strain As1

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We tested the hypothesis that during metabolism of naphthalene and other substrates by *Pseudomonas* sp. strain As1 oxidative stress arises and can be reduced by antioxidant enzymes. Our approach was to prepare plasmid constructs that conferred expression of two single antioxidant enzymes [Fpr (ferredoxin-NADP⁺ reductase) and SOD (superoxide dismutase)] and the pair of enzymes SOD plus AhpC (alkyl hydroperoxide reductase). The *fpr*, *sodA* and *ahpC* genes were placed under the transcriptional control of both the constitutive *lac* promoter and their respective native promoters. Both HPLC and growth-rate analyses showed that naphthalene metabolism was enhanced in the recombinant strains. All antioxidant-overexpressing recombinant strains, with the exception of one with an upregulated *sodA* gene due to the *lac* promoter [strain As1(*sodA*)], exhibited resistance to the superoxide generating agent paraquat (PQ). The growth of strain As1(*sodA*) was inhibited by PQ, but this growth defect was rapidly overcome by the simultaneous overproduction of AhpC, which is a known hydrogen peroxide scavenger. After PQ-induced oxidative damage of the [Fe–S] enzyme aconitase, recovery of enzyme activity was enhanced in the recombinant strains. Reporter strains to monitor oxidative stress in strain As1 were prepared by fusing *gfp* (encoding green fluorescent protein, GFP) to the *fpr* promoter. Growth on salicylate and naphthalene boosted the GFP fluorescent signal 21- and 14-fold, respectively. Using these same oxidative stress reporters, overexpression of *fpr* and *sodA* was found to considerably reduce PQ-induced stress. Taken together, these data demonstrate that the overproduction of Fpr or SodA contributes to oxidative tolerance during naphthalene degradation; however, elevated SOD activity may trigger the generation of excess hydrogen peroxide, resulting in cell death.

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INTRODUCTION

Naphthalene and its metabolites may be toxic to both eukaryotic and prokaryotic cells (Greene *et al.*, 2000; Price *et al.*, 2000; Stohs *et al.*, 2002), and even to naphthalene-metabolizing micro-organisms (Ahn *et al.*, 1998; Garcia *et al.*, 1998; Murphy & Stone, 1955; Park *et al.*, 2004; Pumphrey & Madsen, 2007). Evidence has begun to accumulate showing that inefficient naphthalene biodegradation, either by stationary-phase cells or in the

presence of toxic compounds, can generate toxic metabolic intermediates or reactive oxygen species (ROS) (Kang *et al.*, 2006; Park *et al.*, 2004). Many iron–sulfur proteins are involved in naphthalene biodegradation (Karlsson *et al.*, 2003). Under oxidative stress conditions, the iron in haem and iron–sulfur proteins can be oxidized, which may lead to protein inactivation (Imlay, 2003). In our previous work (Kang *et al.*, 2006), the antioxidants ascorbate and ferrous ion were found to lessen the inhibitory effects of ROS generated during naphthalene metabolism.

Among the microbial defence systems against oxidative stress are the antioxidant enzymes superoxide dismutase (SOD) and ferredoxin-NADP⁺ reductase (Fpr). SOD

Abbreviations: AhpC, alkyl hydroperoxide reductase; Fpr, ferredoxin-NADP⁺ reductase; PQ, paraquat; ROS, reactive oxygen species; SOD, superoxide dismutase.

catalyses the conversion of superoxide to H_2O_2 and O_2 (Giro *et al.*, 2006; Hausladen & Fridovich, 1994). Fpr mediates reversible redox reactions between a single molecule of $\text{NADP}^+/\text{NADPH}$ and two molecules of one-electron carriers, such as ferredoxin or flavodoxin (Bianchi *et al.*, 1995; Lee *et al.*, 2006b). Fpr is involved not only in scavenging ROS but also in the repair of iron-sulfur centres under oxidative stress conditions. Djaman *et al.* (2004) reported that *Escherichia coli* mutants lacking SOD or Fpr repaired their iron-sulfur centre-containing enzymes more slowly than the wild-type strain. Thus, the *fpr* gene confers resistance to oxidative stress in some bacteria (Giro *et al.*, 2006; Lee *et al.*, 2006b). It has been reported that both *fpr* and *sodA* are inducible under oxidative stress in *Pseudomonas* strains (Kim *et al.*, 2000; Lee *et al.*, 2006b). Therefore, our hypothesis is that the overexpression of these antioxidant enzymes may be effective in scavenging the ROS generated during naphthalene degradation. To gain insights into the roles of antioxidant enzymes in the defensive mechanism against oxidative stress, the *sod*, *fpr* and *ahpC* genes were cloned into plasmid vectors that can replicate in *Pseudomonas* sp. strain As1, which was recently isolated from a pollutant-contaminated site (Kang *et al.*, 2006). In addition, reporter strains prepared by fusing the green fluorescent protein gene (*gfp*) to the *fpr* promoter region were used to confirm that oxidative stress occurs during growth on naphthalene and can be lessened by overexpression of Fpr and SOD.

METHODS

Bacterial strains, culture conditions and DNA manipulation.

Bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were grown at 37 °C (*E. coli*) or 30 °C (*Pseudomonas* sp. strain As1) in Luria-Bertani (LB) broth or minimal salts basal medium (MSB; Stanier *et al.*, 1966) with vigorous aeration by shaking at 220 r.p.m. MSB medium was supplemented with different carbon sources [naphthalene (0.5%, w/v), salicylate (2 mM), glucose (2 mM) or pyruvate (2 mM)]. When required, antibiotics were added at the following concentrations: 100 µg ampicillin ml^{-1} ; 100 µg kanamycin ml^{-1} ; 50 µg tetracycline ml^{-1} . Paraquat (PQ) was added at 300 µM. Growth was monitored by measuring the OD_{600} of the cultures using a Biophotometer (Eppendorf). Plasmid isolation, gel electrophoresis, transformation and PCR DNA amplification were performed as described previously (Kang *et al.*, 2006).

Construction of recombinant strains overexpressing antioxidant enzymes and reporter strains.

The fragments encompassing the entire *fpr* (945 bp) and *sodA* (754 bp) genes of *Pseudomonas putida* KT2440 were amplified. The primer pairs used in PCR reaction were as follows. For As1(*fpr*) overexpression, Fpr-OF1/yt2 (CGCAAGCTTGGCGACGAAGACTTGCATTGACG/CGCTCGAG-GCCTGCGCCTTACTTCTCGAC, 945 bp); for As1(*fpr*-R) overexpression, Fpr-OF2/yt2 (CGCGGTACCGGCGACGAAGACT-TGCATTGACG, 945 bp); for As1(*sodA*) overexpression, *sodA*-F/R (CGCAAGCTTCTGCCAAGCCGGATGTAAC/CGCGGATCCGC GC-CAGCCAGCAAAAG, 754 bp); for As1(*sodA*-R) overexpression, *sodA*-F/R2 (CGCGTTCGACGCGCCAGCCAGCAAAAG, 754 bp). The PCR products were digested with *Hind*III/*Xho*I for *fpr*, *Kpn*I/*Xho*I for *fpr*-R, *Hind*III/*Bam*HI for *sodA* and *Hind*III/*Sal*I for *sodA*-R. A broad-host-range promoter-probe vector, pBBR1MCS2 (Kovach *et al.*, 1995),

was used for constructing the recombinant plasmids, pBBRfpr, pBBRfpr-R, pBBRsodA and pBBRsodA-R (Fig. 1a). In this expression plasmid, *fpr* was placed under control of both the *lacZ* and *fpr* promoters (pBBRfpr) or only under the control of the native promoter (pBBRfpr-R). Similarly, *sodA* was placed under control of both the *lacZ* and *sodA* promoters (pBBRsodA) or only under the control of the native promoter (pBBRsodA-R). Each plasmid was transformed into *E. coli* TOP10. Conjugation was performed by triparental filter mating with *E. coli* HB101(pRK2013) (Figurski & Helinski, 1979) and *Pseudomonas* sp. strain As1, thus creating strains As1(*fpr*), As1(*fpr*-R), As1(*sodA*) and As1(*sodA*-R). The transconjugants were selected on LB agar plates, containing 100 µg ampicillin ml^{-1} and 100 µg kanamycin ml^{-1} , at 30 °C.

The *ahpC* (924 bp) fragment was obtained from *P. putida* KT2440 using PCR primer pair *ahpC*-F/R (CGCGAATTCAGCCCCCTCC-TTGAATGTCG/CGCGGATCCGCTTTGGCGGCTGCTTACC, 924 bp). The PCR product was digested with *Eco*RI/*Bam*HI, and then ligated into pBBRsodA, yielding pBBRsodAahpC (Fig. 1a). The constructed plasmid was introduced into strain As1.

Reporter strains to monitor oxidative stress were prepared by fusing the *gfp* gene to the *fpr* promoter. The promoter region of the *fpr* gene, including the gene encoding FinR, a regulator protein, from *Pseudomonas syringae* DC3000 was amplified with the Pfinr-gfp1/Pfinr-gfp2 primer pair (CGCGAATTCGGCCATCGACCGCATTTCT/CGCGGTACCTGGACGCTGAGGACACG) (yielding a 1234 bp fragment), and the fragment was cloned into the *Eco*RI and *Kpn*I cloning site of the pRK415gfp vector (Yin *et al.*, 2003), generating pDCFPrgfp. Conjugation was performed by triparental filter mating with helper strain *E. coli* HB101(pRK2013) and the above-described recombinant strains. The transconjugants were selected on LB agar plates, containing kanamycin (100 µg ml^{-1}) and tetracycline (50 µg ml^{-1}), at 30 °C. Transformation of the plasmid was confirmed by antibiotic resistance and PCR, using appropriate primer pairs (data not shown).

Northern blot analysis. Total RNA of cells cultured with or without PQ (300 µM) was extracted at the early-exponential phase (OD_{600} ~0.3) and isolated using an RNeasy kit (Qiagen), following the manufacturer's instructions. RNA concentrations were estimated by absorbance at 260 nm. Samples of total RNA (10 µg) were loaded on denaturing agarose gels containing 0.25 M formaldehyde, separated, and stained with ethidium bromide to visualize 23S and 16S rRNA. The fractionated RNA was transferred to nylon membranes (Schleicher & Schuell) using a Turboblotter (Schleicher & Schuell). The amount of *fpr* mRNA was determined by hybridizing the membrane with a specific ^{32}P -labelled probe (Invitrogen) prepared by PCR amplification from *P. putida* strain KT2440 with the primer pair *fpr*-K01/*fpr*-K02 (CGCGAATTCAGGCGTTTGGTTTCTCAC/CGCGAATTCAGCCCTGCCGCTTCTCC, 356 bp). The probes for *sodA* and *ahpC* mRNA were prepared by PCR amplification with the primer pairs *sodA*-F/*sodA*-R [primers for As1(*sodA*) overexpression] and *ahpC*-F/*ahpC*-R, respectively. The band intensity of Northern blot data was measured using a densitometry instrument (800 × 1600 d.p.i., UMAX, UTA 2100XL, USA) as previously described (Kim *et al.*, 2006b).

HPLC analysis. A saturated (30 p.p.m.) aqueous solution of naphthalene was used for HPLC analysis. Saturated naphthalene solution was mixed (1:1 ratio) with 2 × MSB medium before adding 10^7 cells ml^{-1} (glucose-grown overnight cultures) to culture media. Residual naphthalene concentration was analysed by HPLC as previously described (Park *et al.*, 2004) except for the following modifications. Samples (0.4 ml) of culture medium were collected at various time points (5 h intervals) and immediately diluted with an equal volume of methanol. Samples were filtered through nylon 66 filters (0.45 µm, 13 mm syringe filter, Whatman). Analytes were

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant markers and characteristics	Reference or source
Bacteria		
<i>Pseudomonas</i> sp. As1	Wild-type, naphthalene degrader, Amp ^r	This laboratory
<i>P. putida</i> KT2440	TOL plasmid-cured derivative of <i>P. putida</i> mt-2	This laboratory
<i>E. coli</i> TOP10	F ⁻ <i>araD139</i> Δ (<i>ara</i> , <i>leu</i>)7697 Δ <i>lacX74 galU galK rpsL</i> (Str ^R) <i>deoR</i> ϕ 80 <i>dlacZ</i> Δ M15 <i>endA1 nupG recA1 mcrA</i> Δ (<i>mrr</i> – <i>hsdRMS mcrBC</i>)	Invitrogen
As1(MCS2)	Strain As1 harbouring pBBR1MCS2	This study
As1(<i>fpr</i>)	Strain As1 harbouring pBBRfpr	This study
As1(<i>fpr</i> -R)	Strain As1 harbouring pBBRfpr-R	This study
As1(<i>sodA</i>)	Strain As1 harbouring pBBRsodA	This study
As1(<i>sodA</i> -R)	Strain As1 harbouring pBBRsodA-R	This study
As1(<i>ahpC</i> <i>sodA</i>)	Strain As1 harbouring pBBRsodAahpC	This study
As1(<i>gfp</i>)	Strain As1 harbouring pDCFPRgfp	This study
As1(<i>gfp</i> , <i>fpr</i>)	Strain As1 harbouring pDCFPRgfp and pBBRfpr	This study
As1(<i>gfp</i> , <i>fpr</i> -R)	Strain As1 harbouring pDCFPRgfp and pBBRfpr-R	This study
As1(<i>gfp</i> , <i>sodA</i>)	Strain As1 harbouring pDCFPRgfp and pBBRsodA	This study
As1(<i>gfp</i> , <i>sodA</i> -R)	Strain As1 harbouring pDCFPRgfp and pBBRsodA-R	This study
Plasmids		
pBBR1MCS2	Km ^r , <i>lacZ'</i>	Kovach <i>et al.</i> (1995)
pBBRfpr	Km ^r , pBBR1MCS2 carrying the <i>P. putida</i> KT2440 <i>fpr</i> gene in the right direction	This study
pBBRfpr-R	Km ^r , pBBR1MCS2 carrying the <i>P. putida</i> KT2440 <i>fpr</i> gene in the reverse direction	This study
pBBRsodA	Km ^r , pBBR1MCS2 carrying the <i>P. putida</i> KT2440 <i>sodA</i> gene in the right direction	This study
pBBRsodA-R	Km ^r , pBBR1MCS2 carrying the <i>P. putida</i> KT2440 <i>sodA</i> gene in the reverse direction	This study
pBBRsodAahpC	Km ^r , pBBRsodA carrying the <i>P. putida</i> KT2440 <i>ahpC</i> gene in the right direction	This study
pRK415gfp	Broad-host-range vector, Tet ^R Mob ⁺	Yin <i>et al.</i> (2003)

separated using a J'sphere ODS-H80 column (150 $\times$ 4.6 mm, YMC) at room temperature with a Younglin model SP930D pump. The mobile phase was 70 % acetonitrile at a flow rate of 1.0 ml min⁻¹. Constituents eluting from the column were detected at 254 nm using a Younglin UV730D detector.

Survival assay. PQ survival experiments were carried out in LB medium, with shaking at 220 r.p.m. at 30 °C. Stationary-phase cultures (50 μ l) were inoculated into 5 ml LB liquid medium at 30 °C with agitation (220 r.p.m.). Then PQ (300 μ M final concentration) was added during the exponential phase (OD₆₀₀ ~0.3–0.5). Cells were harvested from each culture at 1 h intervals and washed with autoclaved phosphate-buffered saline (PBS; pH 7.5). The harvested cells were diluted and serially plated on LB agar. Agar plates were incubated, lid down, at 30 °C for 16 h before colonies were counted.

Enzyme assays. Catalase activity was measured by monitoring the decrease in A₂₄₀ resulting from the elimination of H₂O₂, using a UV-visible spectrophotometer (Optizen 2120, Mecasys, Korea). The absorption coefficient (ϵ) for H₂O₂ at 240 nm was 43.6 M⁻¹cm⁻¹ (Beers & Sizer, 1952). The standard reaction mixture for the assay contained 50 mM potassium phosphate buffer (pH 7.2), 20 mM H₂O₂ and 20 μ l crude extract in a total volume of 3.0 ml. The reaction was performed at 25 °C. The amount of enzyme activity that decomposed 1 μ M H₂O₂ min⁻¹ was defined as 1 unit (U) of activity (Beers & Sizer 1952). Superoxide dismutase (SOD) activity staining was performed as described previously (Beauchamp & Fridovich, 1971; Kang *et al.*, 2006). Supernatants of cell extracts were loaded onto a 7 % native polyacrylamide gel in a running buffer made of 25 mM Tris and 192 mM glycine. Proteins (15 μ g samples) were resolved at 20 mA for 2 h. Subsequently, the gels were processed for SOD activity. The gels were first soaked in 2.5 mM nitro blue tetrazolium (NBT) for 10 min in darkness under gentle shaking. They were then incubated in 50 mM potassium phosphate buffer (pH 7.2)

containing 28 mM N,N,N',N'-tetramethylethylenediamine (TEMED) and 28 μ M riboflavin for 15 min, in the dark and under constant agitation. The SOD activity appeared as a white band on the blue background gel. Aconitase activity (Giro *et al.*, 2006) was measured at 25 °C in 90 mM Tris/HCl, pH 7.5, 20 mM sodium isocitrate, by following the formation of *cis*-aconitate at 240 nm (ϵ_{240} = 3.6 mM⁻¹ cm⁻¹), using a UV-visible spectrophotometer (Optizen 2120, Mecasys). The wild-type and recombinant strains were treated with PQ for 30 min, then transferred to PQ-free LB medium and the aconitase activity measured in a time-dependent manner.

Quantification of GFP fluorescence. Bacterial cells containing *gfp* plasmids (Table 1) were grown in LB broth (+ 300 μ M PQ) or MSB medium supplemented with glucose (2 mM), pyruvate (2 mM), salicylate (2 mM) or naphthalene (0.5 %). After exposure to PQ for 3 h, a 2 ml cell suspension was collected using a microcentrifuge (13 000 r.p.m.) and washed twice with 1 ml PBS. The optical density of resuspended cultures was then measured and GFP fluorescence intensities were quantified using a microtitre plate reader (VICTOR3, BioRad). The reporter strain expresses a stable GFP variant that absorbs at 488 nm. The microscope was equipped with a fluorescence filter cube for detecting GFP (filter set 38 GFP, Carl Zeiss). The AxioVision software was used to acquire images.

RESULTS

Construction of recombinant strains overexpressing antioxidant enzyme

DNA fragments covering the entire *fpr* or *sodA* genes of *P. putida* KT2440 were amplified from *P. putida* strain KT2440 by PCR. The *fpr* and *sodA* genes were individually

Fig. 1. Construction and characterization of *Pseudomonas* sp. As1, used to test hypotheses in this investigation. (a) Schematic representation of *fpr*, *sodA* and *ahpC* genes inserted into plasmid vectors. Arrows indicate the promoter regions (*PlacZ*, *Pfpr*, *PsodA* and *PahpC*). Each gene was expressed under the control of an external (*PlacZ*) or a native promoter (*Pfpr*, *PsodA* or *PahpC*). (b) Northern blot analyses of *fpr* and *sodA* levels in cells placed under oxidative stress by PQ. (c) Northern blot analysis of *nahAc* expression levels in cells grown on naphthalene as carbon source. Total RNA from cells cultured with or without PQ (300 μ M) was extracted at the early-exponential phase ($OD_{600} \sim 0.3$). The lower panel indicates extraction efficiency of 23S and 16S RNA.

placed under the control either of the constitutive *lac* promoter, to express high amounts of each enzyme constitutively, or of their native promoters, to make them inducible under oxidative conditions (Fig. 1a). The expression of these antioxidant enzyme genes in each recombinant strain was confirmed by Northern blot analysis (Fig. 1b). As expected, the plasmids led to elevated expression of both *fpr* and *sodA*. *fpr* expression was clearly highest with the *lac* promoter, while expression of *sodA* was high with both promoters (Fig. 1b). The addition of PQ induced both genes (Fig. 1b). The transcript levels of

strains As1(*fpr*) and As1(*fpr*-R) grown in the presence of PQ increased ~ 1.9 - and ~ 1.8 -fold compared to cells without PQ. Strain As1(*sodA*) expressed more *sodA* (~ 1.6 -fold), compared to strain As1(*sodA*-R) in LB medium. In the presence of PQ, *sodA* expression in strain As1(*sodA*-R) increased ~ 1.7 -fold, compared to cells grown in LB medium. However, PQ addition only slightly induced *sodA* expression in strain As1(*sodA*), probably because high expression of *sodA* already prevailed in the absence of PQ. The data showed that the *fpr* and *sodA* genes were highly inducible by PQ. A much lower mRNA level of indigenous *sodA* and *fpr* from wild-type strain As1 was detected, possibly due to the probes, which were designed based on the genes present in *P. putida* KT2440, whose genome has been completely sequenced (NCBI, NC002947).

The growth rate of cells overexpressing antioxidant enzymes was measured during naphthalene metabolism. Cells were grown at 30 $^{\circ}$ C in MSB medium supplemented with 0.5 % naphthalene. The growth rates (h^{-1}) of the wild-type (As1) and four recombinant strains [As1(*fpr*), As1(*fpr*-R), As1(*sodA*) and As1(*sodA*-R)] were 0.17 ± 0.01 , 0.29 ± 0.02 , 0.23 ± 0.04 , 0.26 ± 0.03 and 0.25 ± 0.04 , respectively. In separate flasks, the rate of aqueous-phase naphthalene metabolism was monitored using HPLC analysis (data not shown). Naphthalene consumption was most rapid (20 % remaining after 15 h) by strains As1(*fpr*) and As1(*sodA*) and slowest for the wild-type (40 % remaining after 15 h). Thus, the growth-rate and HPLC analyses indicated that naphthalene degradation efficiency was improved in all recombinant cells, compared to the wild-type. In addition, the expression of the *nahAc* gene (encoding naphthalene dioxygenase) was increased in all recombinant cells (Fig. 1c). The *nahAc* mRNA levels of strains As1(*fpr*), As1(*fpr*-R), As1(*sodA*) and As1(*sodA*-R) increased ~ 3.0 -, ~ 3.2 -, ~ 2.9 - and ~ 3.1 -fold compared to wild-type, respectively. This was consistent with previous results showing that the *nahAc* expression levels in *Pseudomonas* sp. strain O1 were highest after the addition of antioxidants (Kang *et al.*, 2006).

Survival of bacterial cells exposed to PQ

The resistance of recombinant cells to PQ, a superoxide generator, was investigated (Fig. 2a). As expected, the parental strain (As1), was inhibited by PQ treatment after 5 h of growth. Strains As1(*fpr*), As1(*fpr*-R) (not shown) and As1(*sodA*-R) (Fig. 2a) were resistant to 300 μ M PQ, as they maintained high growth rates and achieved high cell densities. Surprisingly, the growth of strain As1(*sodA*) was also severely inhibited by the addition of PQ (Fig. 2a).

To further explore whether SOD overproduction was the basis for inhibition of cells in the presence of PQ, the sensitivity of our strains to 300 μ M PQ was tested on LB plates (Fig. 2b). Strain As1 harbouring an empty vector (pBBR1MCS2) was used as a control. The wild-type cells, and strains As1(pBBR1MCS2) and As1(*sodA*) were

Fig. 2. Growth of cells exposed to oxidative stress. (a) Effect of PQ on the growth of strains *As1* (wild-type) and *As1(sodA)* in LB medium. After an initial growth period of 5 h, PQ was added at a concentration of 300 μM (arrow). Bacterial growth was monitored by measuring OD₆₀₀. Treatment designations: no PQ, solid lines; PQ, dotted lines. Strain designations: ◆, wild-type (*As1*); ▲, *As1(sodA)*; ○, *As1(sodA-R)*. The results are means ± SD ($n=3$). (b) Tolerance of cells to PQ. Six strains were grown for 16 h on LB agar in the absence (left) or presence (right) of 300 μM PQ. (c) Survival of wild-type and three recombinant strains in LB medium containing 300 μM PQ. Cells were grown to exponential phase in LB medium and exposed to 300 μM PQ. ◆, Wild-type (*As1*); ▲, *As1(sodA)*; ○, *As1(sodA-R)*; ■, *As1(ahpC_{sodA})*. The results are means ± SD ($n=3$). (d) Northern blot analyses of *ahpC* transcription levels in cells placed under oxidative stress by PQ.

sensitive to PQ, as no growth was observed on the plate, while strains *As1(fpr)* and *As1(fpr-R)* were resistant to PQ. Survival of wild-type and recombinant cells exposed to PQ was assessed in LB medium (Fig. 2c): growth of strain *As1(sodA-R)* was not affected by PQ (Fig. 2c), whereas death of the wild-type and *As1(sodA)* strains occurred in the presence of PQ, possibly due to accumulation of the SOD by-product, hydrogen peroxide.

We used strain *As1(ahpC_{sodA})* (Fig. 1a) to test the hypothesis that PQ-induced death of strain *As1(sodA)* was

due to excess intracellular hydrogen peroxide. We reasoned that overexpression of alkyl hydroperoxide reductase (AhpC) would consume peroxides, thereby compensating for SOD overactivity. We constructed strain *As1(ahpC_{sodA})* overexpressing AhpC and confirmed this strain using Northern blot analysis. As expected, the inserted *ahpC* gene was overexpressed in both the absence and presence of PQ (Fig. 2d). Indeed, the data from strain *As1(ahpC_{sodA})* (Fig. 2c) show that overexpression of AhpC rendered cells more resistant to PQ than strain *As1(sodA)*.

Analysis of SOD, catalase and aconitase activities

In order to confirm the above-shown relationships between SOD and alkyl hydroperoxide reductase, these enzymes were measured directly. Elevated SOD activity was measured in strains As1(sodA-R) and As1(sodA), which have additional copies of the SOD gene. After growth in LB medium without exposure to PQ, the wild-type and strains As1(fpr), and As1(fpr-R) showed low levels of SOD activity (Fig. 3a). However, as expected, the SOD activity in strains As1(sodA-R) and As1(sodA) was substantially increased (Fig. 3a): for strain As1(sodA), the increase was ~4.3-, ~3.0- and ~3.7-fold [compared to wild-type, As1(fpr), and As1(fpr-R), respectively] and for strain As1(sodA-R), the increase was ~2.7-, ~1.9- and ~2.3-fold [compared to wild-type, As1(fpr) and As1(fpr-R), respectively]. The additional band exhibiting SOD activity occurred because of the higher mass of the protein produced by the introduced *sod* gene. In the presence of PQ, strains As1(sodA) and As1(sodA-R) showed even higher SOD expression (Fig. 3a), likely alleviating the oxidative stress generated by PQ. The SOD levels of strains As1(fpr), As1(fpr-R), As1(sodA) and As1(sodA-R) increased ~4.6-, ~3.9-, ~10.5- and ~9.8-fold, respectively, compared to wild-type.

Expression of SOD and catalase were examined in both wild-type and recombinant strains grown on naphthalene (Fig. 3b). Interestingly, both the SOD and catalase activities were highly induced in all recombinant strains during naphthalene metabolism (Fig. 3b). The SOD expression of strains As1(fpr), As1(fpr-R), As1(sodA) and As1(sodA-R) was induced ~1.4-, ~1.4-, ~2.4- and ~2.1-fold compared to wild-type. These results indicate that elevated levels of these enzymes likely routinely counteract the oxidative stress generated during naphthalene metabolism.

Aconitase is a dehydratase enzyme containing a [4Fe-4S] cluster. Aconitase was chosen to investigate whether overexpressed Fpr was involved in the recovery of oxidative damage. Recovery of aconitase activity in all recombinant strains was accelerated compared to the wild-type (Fig. 3c). The aconitase activities of strains As1(fpr) and As1(sodA-R) returned to their initial levels very rapidly – within 10 min. The aconitase activities of strains As1(fpr-R) and As1(sodA) were also regenerated to about 80% of their original levels within 20 min of incubation. The wild-type strain showed a very slow aconitase activity regeneration rate (~50% of the original value in 30 min) after being challenged by oxidative stress.

Responses of oxidative-stress reporter strains (*fpr-gfp* fusions) under different carbon source and oxidative stresses

The effect of different carbon sources on the degree of oxidative stress in *Pseudomonas* sp. strain As1 was examined using several GFP-fused reporter strains. This reporter assay is based on the inducibility of the *fpr* promoter in *P. syringae* DC3000. Previously, our data

Fig. 3. Expression of enzymes in five strains of *Pseudomonas* sp. strain As1 under a variety of oxidative stress conditions. (a) SOD activity in wild-type cells and four recombinants exposed to 300 μ M PQ. After the PQ treatment, the cells were incubated in LB medium at 30 $^{\circ}$ C for 1 h. The photograph shows results of the SOD staining reaction. Arrows indicate overproduced recombinant SOD. (b) SOD and catalase activities of exponentially grown wild-type cells and four recombinant strains grown in MSB with naphthalene as the sole carbon source. The upper panel shows a non-denaturing 7% polyacrylamide gel stained for SOD activity. Each lane was loaded with 15 μ g protein. Arrow indicates overexpressed recombinant SOD. The lower panel shows catalase activity. (c) Recovery of aconitase activity after exposure to PQ. Exponentially grown wild-type and recombinant strains were exposed to 300 μ M PQ. After 30 min treatment, the cells were washed and incubated in fresh LB medium at 30 $^{\circ}$ C. $\blacklozenge$, Wild-type (As1); $\blacktriangle$, As1(fpr); $\blacksquare$, As1(fpr-R); $\bullet$, As1(sodA); $\circ$, As1(sodA-R). The results are means $\pm$ SD ($n=3$).

showed that the *fpr* promoter of *P. syringae* DC3000 is highly inducible in an oxidant-concentration-dependent manner (Lee *et al.*, 2006b). The normalized GFP intensity

(GFP intensity/OD₆₀₀; Lee *et al.*, 2006a) of the wild-type *Pseudomonas* sp. strain As1 cells grown in naphthalene-amended medium increased markedly (~14-fold) compared to pyruvate- or glucose-grown cells (Fig. 4a). The GFP level in salicylate-amended media also increased ~21-fold. GFP fluorescence was also measured in wild-type and recombinant strains during naphthalene degradation. The GFP intensity of all recombinant cells was below that of wild-type (Fig. 4b). This result shows that cells of strain As1 exhibit oxidative stress during naphthalene or salicylate degradation. GFP expression levels were measured in cells overexpressing antioxidant enzymes treated with PQ (Fig. 4c). The GFP level in the wild-type showed a significant increase compared to all the recombinant strains in the absence of PQ (Fig. 4c, white bars), indicating that overexpressed antioxidant enzymes play a role in detoxifying the ROS generated under normal growth conditions. The GFP levels of the wild-type and strain As1(sodA) were dramatically increased in the presence of PQ compared to the other recombinant strains, as shown by GFP intensity patterns in fluorescence photomicrographs (Fig. 4c).

DISCUSSION

ROS may be an unavoidable intracellular byproduct of aromatic-hydrocarbon biodegradation by micro-organisms. The ROS generated during naphthalene degradation (e.g. via enzyme inefficiencies and/or dioxygenase inhibition) may diminish cell growth (Kang *et al.*, 2006; Kim *et al.*, 2006a; Lee, 1999) and simultaneously lead to the expression of native antioxidant enzymes such as those examined here. In our previous study using *Pseudomonas* sp. strain O1 (Kang *et al.*, 2006) we demonstrated that SOD and catalase activities were higher in naphthalene- vs glucose-grown cells. These studies indicate that cells have defence systems that partially guard against oxidative stress.

Furthermore, addition of two antioxidants (ascorbate and ferrous ion) to cells growing on naphthalene had three effects: (i) accelerated cell growth; (ii) increased rate of naphthalene consumption; and (iii) higher expressed levels of naphthalene dioxygenase (*nahAc*) transcripts (Kang *et al.*, 2006). The present study confirmed and extended the work of Kang *et al.* (2006) by demonstrating elevated SOD and catalase levels in naphthalene-grown cells (Fig. 3) and GFP intensity in oxidative-stress reporter strains of *Pseudomonas* sp. strain As1 grown on naphthalene and salicylate (Fig. 4). Strain As1 was chosen in this study because we have shown that it grows well in the presence of both naphthalene and salicylate (Kang *et al.*, 2006). Furthermore, strain As1 has higher tolerance to salicylate than many other isolates. This property may be an important feature in microbial biodegradation.

Salicylate is known to cause uncoupling of oxidative phosphorylation (Saxena *et al.*, 1995), and is also toxic as a naphthalene metabolite (Kang *et al.*, 2006; Price *et al.*,

Fig. 4. Expression of *fpr* promoter-*gfp* fusions of *Pseudomonas* sp. strain As1 in cells grown under various conditions. (a) The wild-type was grown in MSB medium containing glucose, pyruvate, salicylate or naphthalene as carbon source. (b) Wild-type and recombinant strains were grown in naphthalene-amended medium. The results in (b) and (c) are means $\pm$ SD ($n=3$). (c) Wild-type and four recombinant strains were grown in LB, without PQ (white bars) and with PQ (black bars). PQ (300 μ M) was added to exponentially growing cells. After the PQ treatment, the cells were incubated for 3 h before fluorescence micrographs were taken (lower panel). RFU, relative fluorescence units: GFP intensity/OD₆₀₀.

2000). In eukaryotes, salicylic acid causes a significant increase in oxygen uptake, which then generates hydrogen peroxide or other ROS (Battaglia *et al.*, 2005). We

hypothesized that the overexpression of antioxidant enzymes (Fpr or SodA) may be effective in relieving oxidative stress generated during naphthalene degradation. Antioxidant enzymes (Fpr or SodA) were overproduced in the naphthalene-degrading bacterium *Pseudomonas* sp. strain As1 (Fig. 1). As we expected, growth rates, naphthalene-degradation efficiency and naphthalene-catabolic gene expression were improved in all recombinant cells, compared to the wild-type (Fig. 1). These data provide physiological evidence that oxidative stress during naphthalene metabolism can be alleviated by the overproduction of antioxidant enzymes.

Treatment of our *fpr* and *sodA* recombinant strains with the superoxide-generating reagent PQ led to strain-specific responses. Overexpression of Fpr [strains As1(*fpr*) and As1(*fpr*-R)] resulted in resistance to PQ (Fig. 2b). Consistent with these results, Fpr overproduction also boosted cell viability in PQ-exposed *E. coli* cells (Bianchi *et al.*, 1995; Giro *et al.*, 2006). By contrast, overexpression of SOD [strain As1(*sodA*)] produced sensitivity to PQ (Fig. 2). SOD overexpression in *E. coli* has produced variable results: beneficial effects against PQ-mediated oxidative damage (Bhattacharya *et al.*, 2004; Goulielmos *et al.*, 2003); but also increased sensitivity to PQ (Scott *et al.*, 1987). A negative effect of SOD overexpression has also been observed in several mammalian systems (Amstad *et al.*, 1991; Costa *et al.*, 1993). When our data showed a negative side effect of SOD (Fig. 2b), we reasoned that the combination of overproduction of SOD protein in strain As1(*sodA*) and exogenous PQ generated high toxic concentrations of hydrogen peroxide, resulting in cell death.

To eliminate the negative effects of SOD overexpression in the presence of PQ, AhpC and SOD were simultaneously overexpressed in strain As1 (Fig. 2c). Rodriguez *et al.* (2000) demonstrated a similar result, where the negative effects caused by SOD overexpression in mammalian cells were neutralized by catalase overproduction. Our results indicated that AhpC likely plays a crucial role in the removal of the hydrogen peroxide generated by SOD overexpression in the presence of PQ. It is likely that superoxide anions produced as a result of PQ treatment created a significant increase in lipid hydroperoxide and/or the production of hydrogen peroxide by cellular SOD. The superoxide anion could easily cause lipid peroxidation, together with the formation of a perhydroxyl radical (Halliwell & Gutteridge, 1999). Protection from these peroxides inside the cell may be facilitated by AhpC, which is involved in the scavenging of intracellular hydrogen peroxide (Seaver & Imlay, 2001) and is also responsible for conversion of lipid hydroperoxides to the corresponding nontoxic alcohols (Halliwell & Gutteridge, 1984; Wang *et al.*, 2006). The experiments here have not addressed the specific mechanism(s) by which AhpC overexpression enhances the survival of strain As1(*sodA*); however, the impact of AhpC in reducing the toxic effects of SOD was clear.

E. coli aconitase is known to be very sensitive to superoxide (Hausladen & Fridovich, 1994). Superoxide oxidizes the $[4\text{Fe-4S}]^{2+}$ in this dehydratase to the unstable $[4\text{Fe-4S}]^{3+}$, which then rapidly degrades to $[3\text{Fe-4S}]^{1+}$, thereby inactivating the enzyme activity or damaging the metabolic pathway (Flint *et al.*, 1993). When oxidants were removed, the activity of the dehydratase could be restored (Flint *et al.*, 1993). Our results indicate that overexpressed Fpr or SOD in recombinant cells can maintain the aconitase activity under oxidative conditions and allow the enzyme to recover its activity (Fig. 3c). A similar result was obtained when an *E. coli* mutant lacking Fpr showed slow recovery of the aconitase activity compared to the wild-type (Giro *et al.*, 2006; Li & Demple, 1996); in contrast, *E. coli* overexpressing Fpr showed dramatically accelerated recovery of aconitase activity to normal levels within a few minutes.

In this study, the overexpression of SOD and Fpr in *Pseudomonas* sp. strain As1 has been shown to enhance both naphthalene degradation and cellular resistance to oxidative stress. We expect that the results of this study will contribute to the development of new strategies for understanding and manipulating the physiology of micro-organisms important for biotechnology and bioremediation.

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