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# Molecular characterization of FinR, a novel redox-sensing transcriptional regulator in *Pseudomonas putida* KT2440

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FinR is required for the induction of *fpr* (ferredoxin-NADP$^+$ reductase) under superoxide stress conditions in *Pseudomonas putida*. Many proteobacteria harbour FinR homologues in their genome as a putative LysR-type protein. Three cysteine residues (at positions 150, 239 and 289 in *P. putida* FinR) are conserved in all FinR homologues. When these conserved cysteines, along with two other cysteine residues present in FinR, were individually mutated to serines, the FinR remained active, unlike SoxR and OxyR in *Escherichia coli*. The results of our *in vitro* DNA-binding assay with cellular extracts showed that FinR binds directly to the *fpr* promoter region. In order to identify the FinR functional domain for sensing superoxide stress, we employed random and site-directed mutagenesis of FinR. Among 18 single amino acid mutants, three mutants (T39A, R194A and E225A) abolished *fpr* induction without any alteration of their DNA-binding ability, whereas other mutants also abrogated their DNA-binding abilities. Interestingly, two mutants (L215P and D51A) appeared to be constitutively active, regardless of superoxide stress conditions. Ferrous iron depletion, ferric iron addition and *fdxA* (ferredoxin) gene deletion also participate in the regulation of *fpr*. These data indicate that FinR has unusual residues for redox sensing and that the redox-sensing mechanism of FinR differs from the well-known mechanisms of OxyR and SoxR.

## INTRODUCTION

Reactive byproducts of oxygen are generated continuously within cells during their growth under aerobic conditions (Cabiscol *et al.*, 2000). These byproducts, referred to as reactive oxygen species (ROS), are toxic to cells owing to their ability to damage many macromolecules inside cells, including DNA, lipids and proteins. When certain proteins are exposed to subinhibitory concentrations of ROS, either modification of amino acid side chains or destabilization of cofactors in the regulatory proteins occurs, which leads to alterations in the protein structures (Imlay, 2003; Stadtman, 1992). Cells employ these structural alterations as signals for the activation of their defence systems. In *Escherichia coli* cells, the SoxR protein, a MerR-type transcriptional factor, activates *soxS* gene expression in response to superoxide or nitric oxide stresses. SoxS is the global transcriptional regulator that controls a variety of oxidative stress defence proteins, including Zwf (glucose-6-phosphate dehydrogenase) and Fpr (ferredoxin-NADP$^+$ reductase); thus, the products of the induced *soxRS* regulon function to prevent or repair oxidative damage. SoxR harbours a redox-active (2Fe–2S) centre, the redox status of which can govern the transcriptional activity of SoxR (Demple, 1996; Hidalgo & Demple, 1994; Pomposiello & Demple, 2000, 2001).

The oxidative stress system has been studied extensively in *E. coli* and *Salmonella typhimurium* (Farr & Kogoma, 1991). The genome of *Pseudomonas putida* KT2440, a model soil micro-organism, has been sequenced (Nelson *et al.*, 2002) but its oxidative stress systems are currently poorly understood. Previously, we reported that the SoxRS regulon does not exist in the genome of *P. putida* KT2440 and that SoxR does not confer oxidative stress defence (Park *et al.*, 2006). The principal function of SoxR in many other micro-organisms is the control of secondary metabolite production, rather than directing oxidative stress defence (Dietrich *et al.*, 2008). Interestingly, we have identified two important possible redox sensors – FinR and HexR – that may control the expression of the *fpr* gene and the *zwf*-1 gene, respectively (Kim *et al.*, 2008; Lee *et al.*, 2006b). These two proteins are critical for the maintenance of proper NADPH levels inside cells. Both of these proteins are under the control of the SoxRS regulon in *E. coli* (Greenberg *et al.*, 1990; Liochev *et al.*, 1994).

The OxyR protein, a LysR-type transcriptional activator, is another type of redox sensor, which has been shown to be

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Abbreviations: GFP, green fluorescent protein; NBT, nitro blue tetrazolium; PQ, paraquat; ROS, reactive oxygen species.

Two supplementary tables and one supplementary figure are available with the online version of this paper.



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relevant to cellular defence against hydrogen peroxide stresses in *E. coli* and *S. typhimurium* (Christman *et al.*, 1989; Tao *et al.*, 1989). OxyR activation occurring in response to $H_2O_2$ is dependent on two cysteine residues (Cys199 and Cys208) (Zheng *et al.*, 1998). OxyR in *P. putida* controls *katA*, *katB* and *ahpC*, which encode three major peroxide-degrading enzymes (Hishinuma *et al.*, 2006). Thioredoxin reductase (*trxB*) is also regulated by OxyR, and further study will be required to elucidate the mechanisms underlying this OxyR regulation clearly (Hishinuma *et al.*, 2008). The LysR-type transcriptional regulators are a large family of regulatory proteins in the proteobacteria, and are known to regulate a variety of genes and gene functions. They feature an N-terminal DNA-binding motif of ~75 aa, and a C-terminal inducer binding domain of ~225 aa (Schell, 1993). The thiol group in the cysteine residues of many transcriptional factors is the key residue associated with redox-sensing, which has also been fairly well characterized in OxyR (Green & Paget, 2004).

The FinR locus is located alongside the *fpr*. Fpr mediates a reversible redox reaction between $NADP^+$/NADPH and electron carriers (Carrillo & Ceccarelli, 2003). We have demonstrated that FinR is essential for the upregulation of *fpr* in response to superoxide stress in *P. putida* KT2440 (Lee *et al.*, 2006b). Further relevant biochemical data have yet to be collected. FinR has been implicated previously as a LysR family transcription regulator. According to alignments of FinR homologues, three cysteine residues are highly conserved in many proteobacteria. These results compelled us to investigate the activation mechanisms of FinR in response to superoxide stress by using a gel shift assay and reporter fusion using a variety of mutants. In order to obtain insight into possible residues required for activation, we conducted random and site-directed mutagenesis of FinR, and screened for mutants that were incapable of activating transcription.

## METHODS

**Bacterial strains, culture conditions and DNA manipulation.** The bacterial strains and plasmids utilized in this study are listed in Supplementary Table S1 (available with the online version of this paper). Bacteria were grown at 37 °C (*E. coli*) or 30 °C (*P. putida*) in LB medium with vigorous aeration. When required, antibiotics were added at the following concentrations: 200 μg rifampicin $ml^{-1}$; 100 μg ampicillin $ml^{-1}$; 50 μg kanamycin $ml^{-1}$; and 15 μg tetracycline $ml^{-1}$.

**Cloning procedures and mutant construction.** The primers utilized in this study are listed in Supplementary Table S2. In order to delete *fdxA*, a 345 bp fragment of its internal region was amplified via PCR using the FdxA-F and FdxA-R primers. The fragment was cloned into the *Eco*RI and *Kpn*I cloning sites of the pVIK112 vector (Kalogeraki & Winans, 1997), yielding pVIK-fdxA. This plasmid was then introduced into *E. coli* S17-1 λ pir. Conjugation was conducted with *E. coli* S17-1 λ pir (pVIK-fdxA) and *P. putida* KT2440-R as the donor and recipient, respectively. Then, the $pRKP_{fpr\ gfp}$ plasmid was introduced via triparental conjugation into Δ*fdxA*, thereby generating *P. putida* KT2440 (ΔfdxA/$pRKP_{fpr\ gfp}$) (Lee *et al.*, 2006a). The 1096 bp fragment of the *finR* full region was amplified by PCR using the primers FinR-CP1 and FinR-CP2. The fragment was then cloned into the *Eco*RI and *Hin*dIII cloning sites of the pUC19 vector (Yanisch-Perron *et al.*, 1985), thereby generating pUC19-*finR*. The constructed plasmid was then introduced into *E. coli* Top10 and *E. coli* Top10 (pRKP*fpr gfp*).

**Mutagenesis.** The pUC19-*finR* plasmid was utilized as a PCR template under conditions designed to induce frequent nucleotide misincorporation. The PCR conditions were identical to those described previously, with some modifications (Cadwell & Joyce, 1992). The mutagenic reaction mixtures contained 7 mM $MgCI_2$, 1 U *Taq* polymerase, and varying concentrations of the four dNTPs (0.2 mM dATP, dGTP and 1.0 mM dCTP, dTTP). The reaction was conducted in a thermal cycler (Eppendorf) for 35 cycles of 94 °C for 45 s denaturation, 60 °C for 45 s annealing, and 72 °C for 45 s extension. Mutated transformants were screened to measure green fluorescent protein (GFP) intensity. Site-directed mutants were constructed via a three step–four primer overlap/extension PCR technique, using Platinum *Pfx* (Invitrogen) (Ho *et al.*, 1989). The first step was prepared via PCR amplification with the primer pairs FinR-CP1/SDM primer-1 and FinR-CP2/SDM primer-2. Each of the PCR products was annealed at the region overlap and extended to form full-length double-strand mutant DNA. The full-length mutant DNA was amplified using primers FinR-CP1/FinR-CP2.

**Microscopic analysis and quantification of GFP fluorescence.** Cells were viewed with a Carl Ziess Axio Imager microscope and GFP fluorescence was quantified as described previously (Kim *et al.*, 2007). One fluorescence unit was defined as (fluorescence intensity of cells−fluorescence intensity of PBS buffer)/($OD_{600}$ of cells).

**Northern blot analysis.** Northern blot analysis was conducted as described previously (Kim *et al.*, 2008). Total RNA was isolated from 2 ml exponentially growing cells. Samples of total RNA (10 μg) were loaded. Probe was prepared via PCR amplification from *P. putida* strain KT2440 with the fpr Pp-hA/fpr Pp-hS primer pair.

**Electrophoretic mobility shift assay (EMSA).** The EMSA was conducted as described previously (Kim *et al.*, 2008). Exponentially growing cells in LB containing ampicillin were harvested. The P*fpr* DNA probe was generated using the fpr pro2/fpr pro3 primer pair. The reaction mixture (15 μl final volume), containing the P*fpr* probe, crude extract, loading buffer and dI-dC (0.1 μg) in binding buffer [10 mM Tris, pH 7.5, 2 mM $MgCl_2$, 10 % (v/v) glycerol and 75 mM KCl], was incubated for 20 min at room temperature. The resulting complexes were analysed by electrophoresis on 6 % polyacrylamide gels.

**Oxidative stress sensitivity assay.** Cells were grown overnight in liquid LB medium and subsequently diluted 100-fold in the same medium. After further incubation until the cells reached exponential phase ($OD_{600}$~0.5), serially diluted cells were spotted on LB agar with or without paraquat (PQ) (5 μM).

**Nitro blue tetrazolium (NBT) assay.** The NBT test was conducted as previously described, with some modifications (Becerra *et al.*, 2003). Bacterial cells in the exponential growth phase ($OD_{600}$~0.5) grown in LB medium were treated with 0.5 mM PQ. After 1 h exposure to PQ, bacterial suspensions (1 ml) were incubated with 0.5 ml 1 mg NBT $ml^{-1}$ for 30 min at 30 °C. Then 0.1 ml 0.1 M HCl was added, and the tubes were centrifuged for 10 min at 1500 ***g***. The separated pellets were treated with 0.4 ml DMSO to extract the reduced NBT. Finally, 0.8 ml PBS (pH 7.5) was added, and the growth was measured at 575 nm. Total protein concentrations were measured via Bradford assays. One unit was defined as $OD_{575}$ (mg protein)$^{-1}$.

**Homology modelling for FinR structure.** A homology model of FinR was generated as described previously (Yeom *et al.*, 2009a). The FinR model used the CrgA of *Neisseria meningitides* (Sainsbury *et al.*, 2009).


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**Protein purification.** FdxA was purified as described previously (Yeom *et al.*, 2009b). All purification steps were performed at 4 °C, using an FPLC system (AKTA FPLC, Unicorn 4.0, Amersham Bioscience). To purify FdxA, *E. coli* cell pellets containing pET-*fdxA* were resuspended in buffer A (50 mM Tris/HCl and 1 mM DTT, pH 7.5) and disrupted by sonication. After removal of cell debris by centrifugation at 12 000 ***g*** for 30 min, the soluble fraction was loaded onto an anion exchange column (1 ml, DEAE-cellulose, Amersham Bioscience) equilibrated with buffer A, and the proteins were eluted with a 20 ml linear gradient of 0–1 M NaCl in Buffer A (pH 7.5). The fractions (0.5 ml each) were collected and concentrated by ultrafiltration with Centricon (2 ml YM-10, Amicon). The concentrates were applied to a Ni-NTA column (1 ml, His-trap, Amersham Bioscience) equilibrated with binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 40 mM imidazole, pH 7.4) and the proteins were eluted with 15 ml elution buffer (20 mM sodium phosphate, 0.5 M NaCl, 250 mM imidazole, pH 7.4). The fractions were dialysed by ultrafiltration with Centricon (2 ml YM-10, Amicon) and stored at −80 °C in 10 % glycerol. After Ni-NTA purification according to the above methods, the fractions of FdxA were applied to a gel-permeation column (Sephacryl S-200 HR 26/60, Pharmacia) and equilibrated with 50 mM Tris/HCl buffer (pH 7.5) containing 150 mM NaCl and 1 mM DTT. The proteins were eluted from the column with the same buffer at a flow rate of 30 ml $h^{-1}$. SDS-PAGE was carried out using 15 % polyacrylamide gels to check the level of expression and purification.

## RESULTS

### Amino acid sequence analyses of FinR homologues

FinR is annotated as a putative LysR-type transcriptional factor and consists of 308 aa; its gene is positioned divergently adjacent to *fprA* in the *P. putida* KT2440 genome. Many bacteria have one FinR homologue in their genomes, and its function has not been studied at all except in our two previous studies, which showed that FinR is required for the induction of the *fpr* gene in response to superoxide and osmotic stresses (Park *et al.*, 2006); however, the mechanism underlying its regulation has yet to be established in detail. Among the well-characterized proteins, the most closely related homologue is CysB, a LysR-type transcriptional regulator which controls the expression of a variety of genes required for sulfur assimilation (Henikoff *et al.*, 1988; Ostrowski & Kredich, 1989). The sequence identity of CysB with FinR is only 23 %. When 30 FinR homologues from the GenBank database were aligned, it appeared that several regions were highly conserved: the N-terminal and central domain regions are more conserved than the C-terminal regions (Fig. 1). FinR from *P. putida* has 81.5 and 64 % amino acid identity with its homologues *Azotobacter vinelandii* and *Aromatoleum aromaticum* EbN1, respectively. The phylogenetic tree constructed via the alignment of the amino acid sequences of FinR homologues from various bacterial lineages demonstrated that the majority of FinR homologues could be detected in the gammaproteobacteria (Supplementary Fig. S1a, available with the online version of this paper). Because the phylogenetic distance from FinR is closer, the *finR* loci were observed to be similar to *P. putida* KT2440. The widespread distribution of the FinR homologues among the proteobacteria, with few exceptions, is evidence of their later evolutionary appearance. Many redox sensors harbour conserved cysteine residues to control their regulation (Green & Paget, 2004). Three cysteine residues (at positions 150, 239 and 289 in the *P. putida* protein) are extremely well conserved, whereas two other cysteines are not conserved (Fig. 1; Supplementary Fig. S1b). We hypothesized that those three cysteine residues might prove important for redox sensing in FinR homologues.

### FinR senses superoxide stress and ferrous iron depletion

In order to determine whether FinR actually senses superoxide stress, we quantified the *fprA* promoter activity via transcriptional fusion with GFP, as described in our previous studies (Lee *et al.*, 2006a). We verified that the expression of the *fprA* promoter is induced by superoxide-generating agents (Fig. 3a). This notion is further supported by the fact that overexpressed SodA from the ectopic plasmid effectively eliminates superoxide, and thereby reduces *fprA* expression under paraquat treatment conditions (Fig. 2a). These data clearly demonstrated that FinR is activated as the result of superoxide stress.

Recently, we have used Western blot analysis to demonstrate that the FprA protein is also profoundly upregulated under iron stress conditions (Yeom *et al.*, 2009a). In order to confirm the relationship between FinR and iron in the regulation of *fprA*, we measured the *fprA* expression levels of both the wild-type and *finR* mutant (Fig. 2b). In the *finR* mutant, *fprA* expression is dramatically reduced under both ferric iron-repleted and -depleted conditions, whereas the wild-type induces *fprA* expression in response to those iron-stress conditions (Fig. 2b). We concluded that the *fprA* expression under iron stress conditions might be associated with FinR.

### Expression of FinR–*fprA* system in a heterologous host, *E. coli*

It is desirable to use *E. coli* to critically assess the residues relevant to FinR activation, owing to the ease with which transformations and gene manipulations can be conducted. The *fprA–gfp* reporter vector coupled with the plasmid in which FinR is constitutively expressed when introduced into *E. coli* Top10 (data not shown). When exposed to PQ, Top10 cells harbouring the two plasmids showed responses similar to those of *P. putida* KT2440 (Fig. 3a). The GFP level in the reporter strains accumulated in cases in which cells were exposed to different PQ concentrations. The greater the concentration of PQ applied, the more *fprA* expression was induced. GFP expression was also visible by using fluorescence microscopy. Without PQ treatment, the reporter strains did not induce GFP expression in LB media (Fig. 3b), whereas high levels of GFP expression were noted


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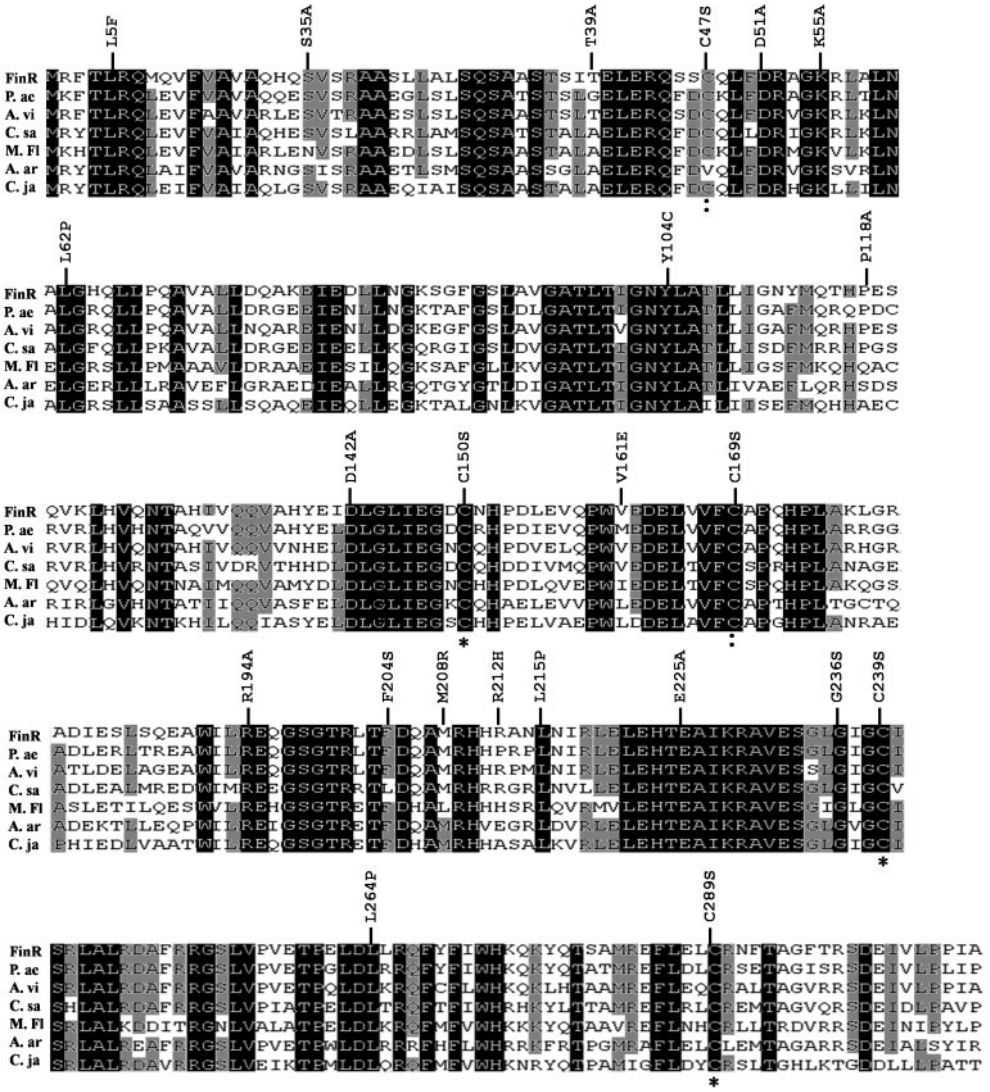


**Fig. 1.** Alignment of FinR homologues from different organisms. Amino acid sequences (obtained from NCBI) were aligned via CLUSTAL W. FinR, *P. putida* KT2440 (AE015451.1); P. ae, *Pseudomonas aeruginosa* PAO1 (AE004091.2); A. vi, *A. vinelandii* DJ (CP001157.1); C. sa, *Chromohalobacter salexigens* DSM 3043 (CP000285.1); M. fl, *Methylobacillus flagellatus* KT (CP000284.1); A. ar, *Aromatoleum aromaticum* EbN1 (CR555306.1); C. ja, *Cellvibrio japonicus* Ueda107 (CP000934.1). Highly conserved residues in seven FinR homologues (black shading), conserved residues in several FinR homologues (grey shading), conserved cysteine residues in all FinR homologues (asterisks) and the rest of the cysteine residues present in FinR (double dots) are highlighted.

in LB media containing 1 mM PQ (Fig. 3b). These data verified that *E. coli* harbours all the necessary systems to induce the *fpr* gene in response to superoxide stress.

## Binding of FinR to the promoter region of the *fpr* gene

The binding activity of FinR to the *fprA* promoter region has never before been studied. As FinR has been shown to be insoluble in overexpression systems, we utilized cell lysates in further binding studies. If the cell extracts from *E. coli* Top10 harboured an empty vector, the promoter probes were not complexed with any proteins (Fig. 3c) thereby confirming that there are no other proteins that can bind to the *fprA* promoter regions in *E. coli* Top10 strains. The EMSA results showed that FinR binds to the *fprA* promoter region (Fig. 3c).

## Site-directed mutagenesis of conserved cysteine residues

Cysteines have been shown to be very important residues in many redox-controlling transcriptional factors. They can be oxidized to several different reversible redox states, including disulfide (R-S-S-R′), sulfenic acid (R-SOH) and


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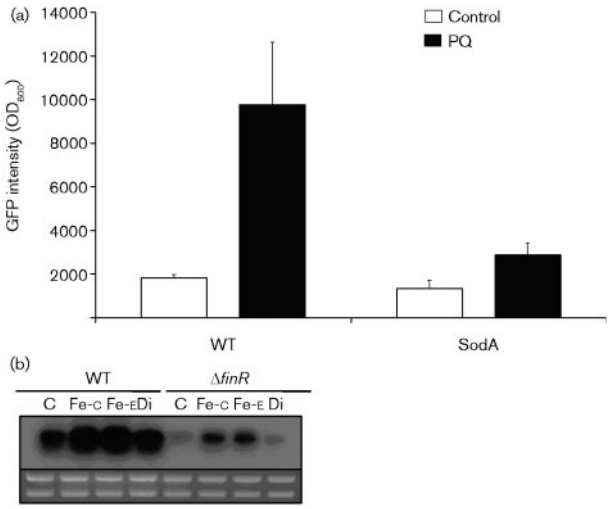


**Fig. 2.** Analysis of *fpr* expression depending on superoxide stress and iron depletion. (a) GFP intensity was measured after PQ shock. Quantification of GFP in wild-type [*P. putida* KT2440 (pRKP$_{fpr\ gfp}$)] and SodA [*P. putida* KT2440 (pRKP$_{fpr\ gfp}$/pBBR *sodA*)]. Samples were treated (black bars) or not (white bars) with 1 mM PQ. Error bars indicate SD of data from three independent experiments. (b) Northern blot analysis of *fpr* transcription. The mRNA induction level of *fpr* genes in minimal medium (M9) with 0.5 μM ferric-citrate is shown. All chemicals were applied to the cells for 30 min at 10 μM. C, no treatment; Fe-C, ferric-citrate; Fe-E, ferric-EDTA; and Di, 2′,2′-dipyridyl.

*S*-nitrosothiol (R-SNO) (Green & Paget, 2004). In order to determine whether the conserved cysteine residues in FinR are relevant to sensing oxidative stress, we mutated each of the three conserved cysteines to serines; the remaining two cysteines were also mutated via site-directed mutagenesis. The transcription activities of those strains containing mutated FinR were then assayed. It appeared that the C46S, C150S, C169S, C239S and C289S mutant strains exhibited transcription activities similar to those of the strain harbouring wild-type FinR (Fig. 4a). Unlike other redox transcriptional factors, those five cysteines are not involved in FinR activation. It was noted that FinR exhibits a redox-sensing mechanism that differs from OxyR (Zheng *et al.*, 1998).

## Characterization of single amino acid mutants

In order to identify the amino acid residues necessary for FinR activation and DNA binding, we conducted random and site-directed mutagenesis of FinR. Random mutagenesis was accomplished via error-prone PCR, and site-directed mutagenesis was achieved via a three step–four primer overlap/extension PCR technique. No *fprA* induction was detected in three mutants (T39A, R194A and E225A; Fig. 4a), although their DNA-binding ability to the target gene remained intact (Fig. 4b). Two mutants were constitutively active (L215P and D51A) regardless of oxidative stress conditions (Fig. 4a). The basal level of *fprA* expression in the L215P mutant was found to be similar to that observed in the wild-type strain following PQ treatment. The transcriptional level of *fprA* in the L215P mutant increased further upon the addition of PQ.

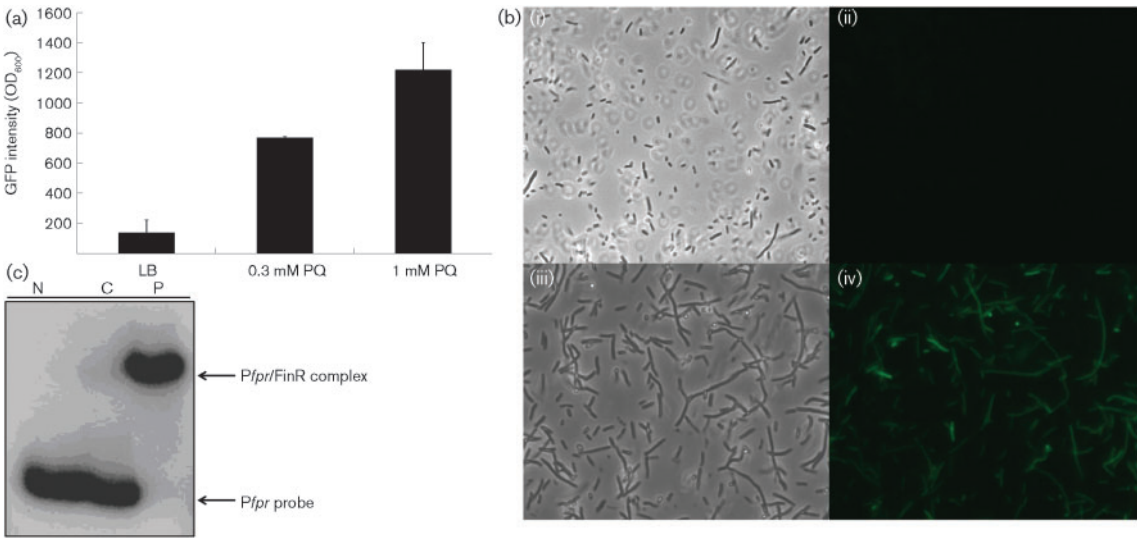


**Fig. 3.** Expression of *P. putida* FinR–*fpr* system in *E. coli.* (a) GFP intensity was measured after PQ shock in *E. coli* Top10 (pRKP$_{fpr\ gfp}$/ FinR). Bars indicate SD of data from three independent experiments. (b) Phase contrast (i) and fluorescence (ii) microscopy of Top10 (pRKP$_{fpr\ gfp}$/ FinR) grown LB media, and phase contrast (iii) and fluorescence (iv) microscopy of the same cells exposed to 1 mM PQ. (c) *In vitro* EMSA. Lanes: N, retardation assay of the P*fpr* probe with no protein; C, P*fpr* probe with cell extract from Top10 harbouring empty vector; P, P*fpr* probe with cell extract from Top10 harbouring FinR expression vector. A 4 % polyacrylamide gel was used.



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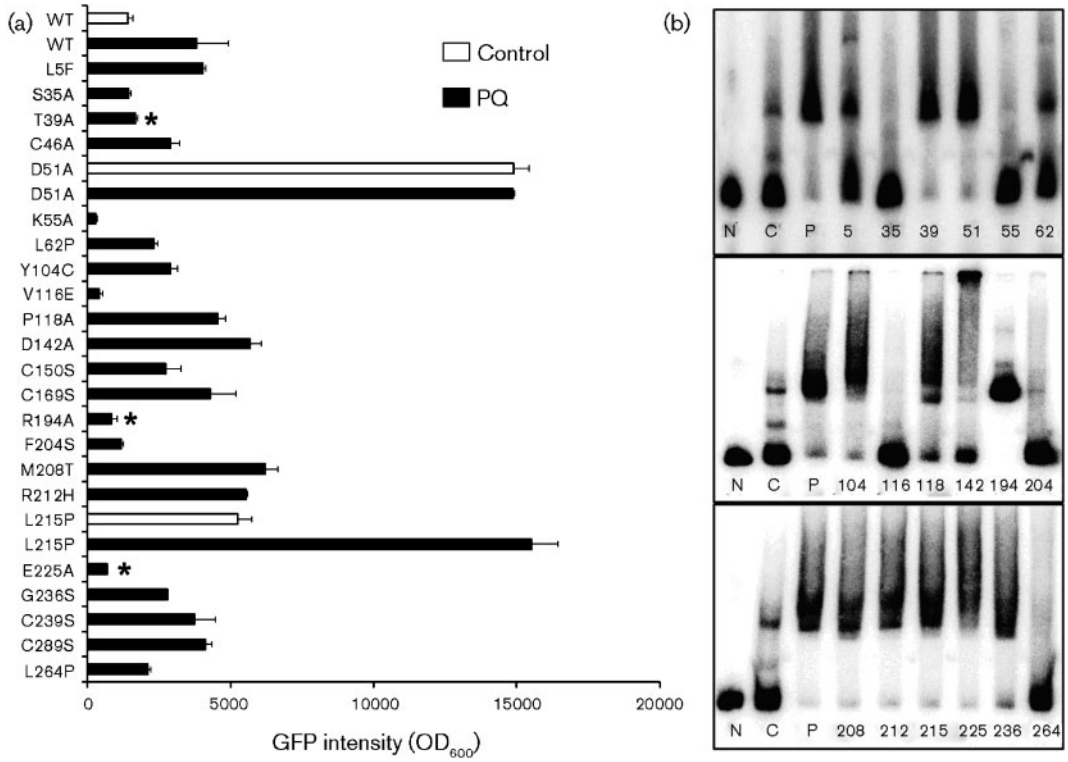


**Fig. 4.** Characterization of amino acid substitutions. (a) Quantification of GFP expression in *E. coli* Top10 harbouring wild-type *finR* and single amino acid mutant *finR*. PQ (1 mM) was added to cells exponentially grown in LB media. Asterisks indicate *finR* mutants with impaired redox sensing. Error bars indicate SD of data from three independent experiments. (b) *In vitro* EMSA. Lanes N–C are the same as in Fig. 3(c). The marked numbers indicate each substituted residue in FinR.

However, the D51A mutant exhibited threefold higher induction levels in both the absence and presence of PQ compared with the wild-type strain. Because five mutants (S35A, K55A, V116E, F204S and L264P) lost their DNA-binding abilities, no *fprA* induction was detected in these strains (Fig. 4b). Two mutants (L5F, L62P) are partially defective in their DNA binding, but their transcriptional activities remain functional (Fig. 4). Transcriptional activities and DNA-binding abilities of all other mutants were similar to those of wild-type in the absence and presence of PQ (Fig. 4). Interestingly, the D142A mutant showed a greater degree of retardation than other proteins (Fig. 4b), although its transcriptional activity remained the same as the wild-type. This mutant might be altered in its multimerization.

## Involvement of iron–sulfur cofactor-containing FdxA in *fprA* expression

Previously, it has been demonstrated that the disruption of FdI, encoding *A. vinelandii* ferredoxin I (*Av*FdI), a homologue of FdxA, results in the overexpression of ferredoxin-NADP$^+$ reductase. In the presence of PQ, the *fprA* activation level in the wild-type strain was identical to that observed in the *fdxA* mutant. The expression level of *fprA* was not increased in the *fdxA* mutant, even after the addition of PQ to the growth medium (Morgan *et al.*, 1988). As these data may shed some light on the issue of *fprA* regulation, we constructed an *fdxA* mutant of *P. putida* KT2440 in an effort to evaluate the effects of *fdxA* mutation. The expression levels of *fprA* in both the wild-type and mutant strains were measured in the presence and absence of PQ. The promoter activity of *fprA* in the mutant was very high, even under normal conditions, compared with the wild-type strain, but *fprA* expression increased even further upon the addition of PQ (Fig. 5a). The transcriptional fusion data were confirmed via Northern blot analysis (Fig. 5b). We speculated that the intracellular superoxide concentration was increased in the mutant, and that the expression of *fprA* increased in a dose-dependent manner. Superoxide anion production in the *fdxA* mutant was measured as described previously (Becerra *et al.*, 2003). Superoxide production in the mutant increased more profoundly under normal growth conditions than in the wild-type strain (Fig. 5c). The pattern of *fprA* expression in the *fdxA* mutant correlated with the superoxide anion concentration. Collectively, our results show that FdxA participates indirectly (or directly) in *fprA* regulation via the production of superoxide inside cells. The results further indicate that *fprA* regulation is a more complicated issue than had been thought previously. Exponentially grown cells were serially diluted and spotted on LB agar,


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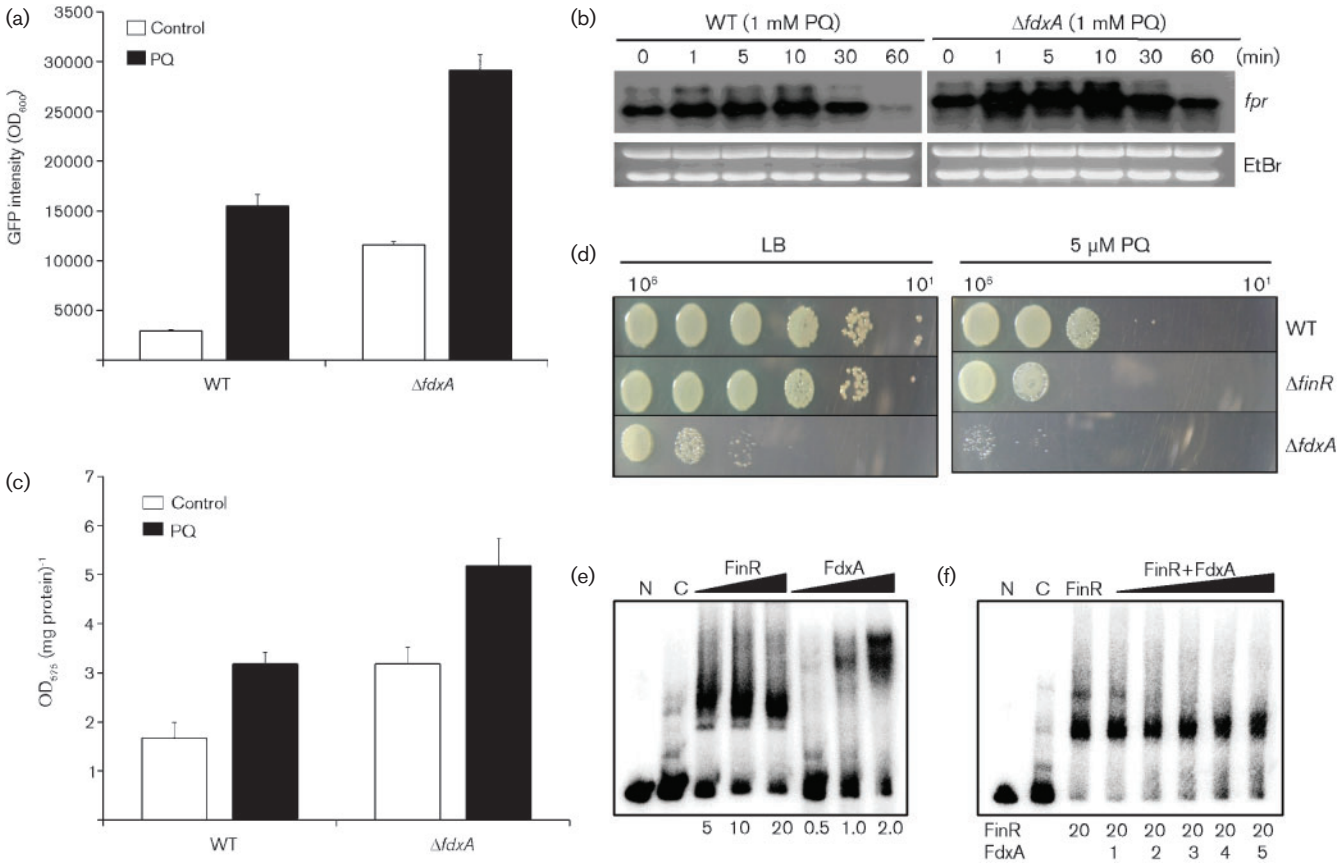


**Fig. 5.** Characterization of *fdxA*. (a) Quantification of GFP expression in wild-type [*P. putida* wild-type (pRKP$_{fpr\ gfp}$)] and Δ*fdxA* [*P. putida* (Δ*fdxA*/pRKP$_{fpr\ gfp}$)] cells. Samples were treated (black bars) or not (white bars) with 1 mM PQ. (b) Northern blot analysis of *fpr* transcription. Total RNA was extracted 1, 5, 10, 30 and 60 min after treatment of cells with 1 mM PQ; 0 min is the normal condition. EtBr, Ethidium bromide. (c) Concentration of intracellular superoxide anion. Samples were treated (black bars) or not (white bars) with 0.5 mM PQ. Error bars indicate SD of data from three independent experiments. (d) Sensitivity assay of *P. putida* wild-type (WT), *finR* and *fdxA* mutant strains to PQ. (e, f) *In vitro* EMSA. Lanes N and C are the same as in Fig. 3(c). FinR, P*fpr* probe with cell extract from *E. coli* Top10 harbouring the FinR overexpression vector; FdxA, purified ferredoxin protein. The amount of protein (μg) is given below the figures.

with and without PQ. The *fdxA* mutant proved more sensitive than the wild-type and FinR mutant strains (Fig. 5d). To investigate whether FdxA binds to the *fprA* promoter region, EMSAs were conducted using purified FdxA proteins. Surprisingly, the EMSA results demonstrated that FdxA retards the migration of the DNA probe (Fig. 5e). The explanation for this observation is not straightforward. To examine the relationship between FinR and FdxA on the *fprA* promoter region, we carried out retardation study using FinR crude extract and FdxA protein. This showed that binding of FdxA to the *fprA* promoter region is blocked by FinR (Fig. 5f).

## DISCUSSION

Interestingly, data regarding the *fpr* regulation system of *A. vinelandii* suggested the involvement of two proteins: ferredoxin I (FdI, a homologue of FdxA, hereafter referred to as FdxA) and the E1 component of pyruvate dehydrogenase complex (PDHE1). It has been demonstrated that PDHE1 binds to a very specific DNA sequence upstream of the *fpr* gene, and that FdxA binds specifically to DNA-bound PDHE1 (Regnström *et al.*, 1999). Disruption of FdxA transcriptionally activates the *fpr* gene to the same extent in response to either the presence or the absence of PQ. A recent report has shown that PDHE1 is not the *fpr*-promoter-binding protein *in vivo* and theorizing that the effect of FdxA on *fpr* expression might occur in an indirect manner (Park *et al.*, 2007). Deletion of *fdxA* may result in a sizeable superoxide burden on cells that leads to the induction of the *fpr* gene (Park *et al.*, 2007). This assumption was strongly supported by our finding that the deletion of FdxA increased superoxide content within the cells (Fig. 5c). The data presented here demonstrated that FdxA binds to the promoter regions



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of the *fprA* gene (Fig. 5e). Because the molecular mass of Fdx is very small and FdxA is not known to be a DNA-binding protein, it seems likely that FdxA assists in the binding of other DNA-binding protein to the *fprA* promoter. We cannot rule out the possibility that purified FdxA solution was contaminated by PDEH1 during purification steps. Interestingly, FdxA binding is inhibited by the presence of FinR (Fig. 5f). Involvement of PDHE1 in FinR activation remains to be assessed.

Among several bacterial redox sensors, OxyR and OhrR utilize mechanisms involving cysteine residue modification. Key cysteine residues in both proteins are oxidized in response to oxidative stress conditions. *Bacillus subtilis* OhrR is a member of the MarR family transcription regulators (Fuangthong *et al.*, 2001). The amino acid sequence of FinR contains five cysteines. Although all FinR homologues harbour conserved cysteines, they do not appear to be thiol-based sensors (Fig. 4a). In this study, we have demonstrated for the first time that FinR is a specic DNA-binding protein (Fig. 3c). We employed random and site-directed mutagenesis to define the regions of FinR relevant to DNA binding and activation. A model of the structure of FinR was established using the structure of CrgA (Fig. 6). CrgA is a member of the LysR-type transcriptional regulatory family, and amino acid similarity between FinR and CrgA is very low (19.8 %). Nevertheless, CrgA is the closest to FinR in terms of protein structure. The CrgA subunit of the full-length protein comprises an N-terminal DNA-binding domain (residues 1–60), connected via a long linker helix (residues 61–89) to a C-terminal regulatory domain. Three mutants (T39A, R194A and E225A) showed low levels of *fprA* induction, but their DNA-binding ability remained unaffected (Fig. 4b). It is worth commenting that two sensing-defective mutants (R194A and E225A) are mapped to the sensing domain (Fig. 6). They might be mapped at or near possible redox-active residues. The mutants L215P and D51A significantly activated *fpr*, even without PQ (Fig. 4a). The D51A mutant is mapped to the helix-turn region in the binding domain (Fig. 6). Based on T39A and D51A, we speculated that changes in the DNA-binding domain could influence the transcriptional activity of FinR. The remaining mutation, L215P, mapped at the sensing domain, might be involved in interactions with RNA polymerase or reductase. This constitutively active FinR might be elucidated further by other similar studies. Constitutively active SoxR could be obtained following the deletion of nine C-terminal amino acids, which might interfere with its interaction with a SoxR reductase (Nunoshiba & Demple, 1994). Among an additional seven mutants, four mutants (L5F, S35A, K55A and L62P) have been mapped to the DNA-binding region consistent with the EMSA results (Figs 4 and 6). The remaining three mutants (V116E, F204S and L264P) are also defective in their DNA binding (Fig. 4b), although they are not mapped to the DNA-binding region. This may be because these mutants have an unstable protein structure.

The OxyR of *Deinococcus radiodurans*, which harbours one conserved cysteine, functions not only as a positive regulator

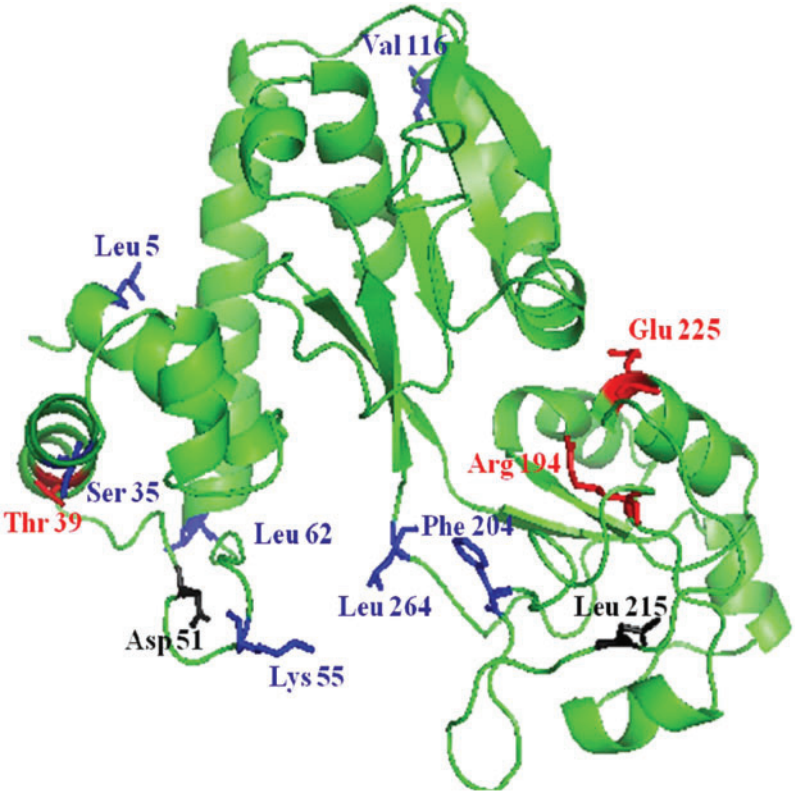


**Fig. 6.** Modelling the structure of FinR in *P. putida*. Positions of single amino acid mutations on the FinR structure are shown. The residues found to be important for redox sensing (T39, R194 and E225) are shown in red. Constitutive mutants (D51 and L215) are shown in black. Mutants that result in defective DNA binding are shown in blue.


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but also as a negative regulator of different classes of genes (Chen *et al.*, 2008). OxyR regulation in other bacteria is more complicated than had previously been anticipated. In a similar fashion, *fprA* gene regulation might be involved intricately with other factors, and FinR appears to utilize a unique redox-sensing mechanism which differs profoundly from other redox-sensing transcriptional regulators.

In summary, our findings demonstrate that FinR of *P. putida* KT2440 performs a crucial role in cellular defences against superoxide and ferrous iron depletion stresses. Additionally, FinR can bind directly to the promoter region of *fprA*. Ferredoxin may also have some influence on the expression of *fprA*.

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