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Pseudomonas aeruginosa OspR is an oxidative stress sensing regulator that affects pigment production, antibiotic resistance and dissemination during infection

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Abstract

Summary—Oxidative stress is one of the main challenges bacteria must cope with during infection. Here, we identify a new oxidative stress sensing and response *ospR* (oxidative stress response and pigment production Regulator) gene in *Pseudomonas aeruginosa*. Deletion of *ospR* leads to a significant induction in H_2O_2 resistance. This effect is mediated by de-repression of *PA2826*, which lies immediately upstream of *ospR* and encodes a glutathione peroxidase. Constitutive expression of *ospR* alters pigment production and β-lactam resistance in *P. aeruginosa* via a *PA2826*-independent manner. We further discovered that OspR regulates additional genes involved in quorum sensing and tyrosine metabolism. These regulatory effects are redox-mediated as addition of H_2O_2 or cumene hydroperoxide leads to the dissociation of OspR from promoter DNA. A conserved Cys residue, Cys-24, plays the major role of oxidative stress sensing in OspR. The serine substitution mutant of Cys-24 is less susceptible to oxidation *in vitro* and exhibits altered pigmentation and β-lactam resistance. Lastly, we show that an *ospR* null mutant strain displays a greater capacity for dissemination than wild-type MPAO1 strain in a murine model of acute pneumonia. Thus, OspR is a global regulator that senses oxidative stress and regulates multiple pathways to enhance the survival of *P. aeruginosa* inside host.

Introduction

Pseudomonas aeruginosa is a Gram-negative bacillus that is ubiquitous in diverse environments. It is also an opportunistic pathogen of humans, most notably those afflicted with cystic fibrosis or whose immune systems have been compromised (Reynolds *et al*., 1975; Cross *et al*., 1983; Govan and Harris, 1986). Similar to many other human pathogens, *P. aeruginosa* must overcome the oxidative stress response generated by phagocytic cells for successful infection. Phagocytes utilize the cytotoxic effects of reactive oxygen species (ROS), such as superoxide, H_2O_2, and hydroxyl radical, to contain bacterial infections. In order to

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counter this innate immune response, *P. aeruginosa* possesses a multifaceted defence system against oxidative stress, with proteins such as catalase and superoxide dismutase, thioredoxin, glutaredoxin, and small molecules such as glutathione and melanin (Hassett and Cohen, 1989; Scandalios, 1997; Rodríguez-Rojas *et al*., 2009).

As key regulators, OxyR and SoxR have been well known to modulate oxidative stress response in bacteria (Storz and Imlay, 1999). Homologues of these two proteins have been identified in many bacterial species, including *P. aeruginosa*. In *P. aeruginosa*, OxyR is important for oxidative stress defence (Ochsner *et al*., 2000) and is required for full virulence in rodent and insect models of infection (Lau *et al*., 2005). Recently, OxyR has also been shown to regulate secretion of potent cytotoxic factors in *P. aeruginosa* (Melstrom *et al*., 2007). On the contrary, *P. aeruginosa* does not rely on SoxR for an oxidative stress response (Kobayashi and Tagawa, 2004; Palma *et al*., 2005). Instead, *P. aeruginosa* SoxR responds to phenazines (Dietrich *et al*., 2006), which act as signalling molecules in the bacterium (Dietrich *et al*., 2006). Phenazines produced by *P. aeruginosa* are colourful, redox-active antibiotics. These compounds have profound effects on the structural organization of colony biofilms (Dietrich *et al*., 2009) and have been identified as virulence factors in a number of *in vivo* model systems (Wilson *et al*., 1988; Mahajan-Miklos *et al*., 1999; Ran *et al*., 2003; Gibson *et al*., 2009). A connection between phenazine biosynthesis and oxidative stress response in *P. aeruginosa* is as yet unclear.

In our previous work, we showed that the MarR family transcriptional regulators MgrA and SarZ play key roles in virulence regulation in *Staphylococcus aureus* using an oxidation-sensing mechanism (Chen *et al*., 2006; 2008a). Both MgrA and SarZ are members of a subfamily of MarR proteins that utilize cysteine oxidation to sense oxidative stress and regulate bacterial responses. The prototype, OhrR in *Bacillus subtilis*, regulates bacterial resistance to organic hydroperoxides (Fuangthong *et al*., 2001; Sukchawalit *et al*., 2001; Mongkolsuk and Helmann, 2002; Newberry *et al*., 2007). However, in a pathogenic bacterium such as *S. aureus*, these regulators seem to play regulatory roles that have much broader and profound effects on global properties of the pathogen. These discoveries in *S. aureus* raise the possibility that the OhrR/MgrA homologues in pathogenic *P. aeruginosa* may also assume global roles through sensing oxidative stress. Here, we present a new redox-active regulator, OspR, in *P. aeruginosa*. This protein, using an oxidation-sensing mechanism, is involved in oxidative stress response, pigment production, β-lactam resistance and dissemination of *P. aeruginosa* during infection. OspR also affects expression of genes involved in tyrosine metabolism (*hmgA, PA2010*) and quorum sensing (*phzM, phzS, PA1897*). These results should help shed light on the multifaceted oxidative stress response in *P. aeruginosa* and contribute to understanding its role in *P. aeruginosa* physiology and pathogenesis.

Results

Identification of S. aureus mgrA *homologous genes in* P. aeruginosa

As demonstrated in our previous work, the global regulator MgrA plays a key role in virulence regulation in *S. aureus* using an oxidation-sensing mechanism (Chen *et al*., 2006). To identify the MgrA homologues in *P. aeruginosa*, we performed BLASTP analyses with *S. aureus mgrA* against the genome of *P. aeruginosa* PAO1. Two hits were obtained with PA2825 showing 37.59% identity to MgrA while PA2849 sharing 31.25% identity with MgrA (Fig. S1A). Pfam analysis indicated that both PA2825 and PA2849 proteins possess the MarR-type helix–turn–helix motif and as such belong to the family of MarR proteins.

Downstream of *PA2849* is *PA2850*, and there exists a 142 bp intergenic region between *PA2849* and *PA2850*. The encoding protein of *PA2850* shows a 78% similarity to Ohr (organic hydroperoxide resistance) protein in *Xanthomonas campestris* pv. *phaseoli*. The gene encoding

OhrR (transcriptional regulator, MarR family) from *X. campestris* pv. *phaseoli* is co-transcribed with its downstream adjacent gene, *ohr* (Panmanee *et al*., 2002; Klomsiri *et al*., 2005). Considering that numerous bacteria maintain this genetic organization of *ohrR* and *ohr* (Fuangthong *et al*., 2001; Sukchawalit *et al*., 2001; Chuchue *et al*., 2006), it is very likely that PA2849 is an OhrR homologue in *P. aeruginosa*.

According to the annotation of *P. aeruginosa* PAO1 genome, *PA2825* is a likely transcriptional regulator gene. The coding regions of *PA2825* and *PA2826* (a putative glutathione peroxidase gene) overlap by four base pairs; the coding region of *PA2825* starts at the fourth nucleotide before the end of *PA2826* (Fig. S1B). Sequence analysis of this genetic organization in *Pseudomonas* species indicates that *PA2825* orthologues are present in various *P. aeruginosa* and *P. fluorescens* strains but absent in *P. putida*, *P. syringae* and *P. entomophila L48* strains (Fig. S2). We focused on *PA2825* and named it as *ospR* based on observed phenotypes presented in this study.

ospR *null mutant and the analysis of* PA2827, PA2826 *and* PA2824 *RNA expression*

To probe the biological functions of *ospR*, we generated an *ospR* null mutant strain (Δ*ospR*, Fig. S1B) through the method of allelic replacement as described in *Experimental procedures*. Further, the complementing plasmid of p-*ospR* (Table 1) was constructed and then transformed into Δ*ospR*, yielding the Δ*ospR*/p-*ospR* strain. Introduction of p-*ospR* into Δ*ospR* provided for constitutive expression of *ospR* without IPTG induction (data not shown). We performed the Northern blot analysis of mRNA of the *ospR* neighbouring genes *PA2827*, *PA2826* and *PA2824* respectively. The Northern blot results showed that there was no significant difference in mRNA levels of *PA2827* and *PA2824* between the Δ*ospR* strain (harbouring pAK1900) and the wild-type MPAO1 strain (harbouring pAK1900) (Fig. 1A). However, a significant overexpression of the *PA2826* transcript was detected in the Δ*ospR* strain as compared with the wild-type strain (Fig. 1A). Complementation with p-*ospR* in the Δ*ospR* strain restored the low mRNA level of *PA2826* similar to that observed in the wild-type strain (Fig. 1A). These results indicate that OspR represses expression of *PA2826*. Interestingly, the deletion of *ospR* in *P. aeruginosa* MPAO1 also resulted in a slight increase of the mRNA level of *PA2850* (*ohr*) (Fig. S3A).

OspR contributes to oxidative stress response through regulation of PA2826

Deletion of *ospR* in *P. aeruginosa* leads to de-repression of *PA2826*, a gene encoding a glutathione peroxidase (GPx) protein based on the annotation of the *P. aeruginosa* PAO1 genome and sequence analysis. The main biological role of glutathione peroxidase is to protect the organism from oxidative damage. In order to assess whether *ospR* plays a role in bacterial response to oxidative stress, we tested the sensitivity of the Δ*ospR* strain to H_2O_2 and paraquat by using stress plate assay as described in *Experimental procedures*. From the assay Δ*ospR* strain exhibited an increased resistance to H_2O_2 (Fig. 1B and Table S1), but showed a hypersensitivity to paraquat when compared with the wild-type MPAO1 strain (Fig. 1C). These phenotypes could be complemented by the introduction of p-*ospR* (Fig. 1B and C, and Table S1).

To examine whether the altered resistance/sensitivity to H_2O_2/paraquat is due to the overexpression of *PA2826* in the Δ*ospR* strain, a plasmid for constitutive expression of *PA2826* (p-*PA2826*) was constructed and then transformed to the wild-type strain. Additional stress plate assays were performed and results are shown in Fig. 1D. The MPAO1/p-*PA2826* strain displayed an increased resistance to H_2O_2, and an increased sensitivity to paraquat when compared with the control strain MPAO1/pAK1900. The increase of H_2O_2 resistance is more evident in a strain overexpressing *PA2826* (p-*PA2826*) than the *ospR* mutant strain (Fig. 1 and

Table S1). This phenotype correlates with the transcription levels of *PA2826* in these stains as shown by Northern blot analysis (Fig. S3).

To further demonstrate that *PA2826* mediates the altered resistance/sensitivity to H_2O_2/paraquat, we generated a *PA2826-ospR* null mutant strain (Δ*PA2826-ospR*), as described in *Experimental procedures*. Stress plate assay indicated no significant difference in resistance/sensitivity to H_2O_2/paraquat between the Δ*PA2826-ospR* strain and the wild-type MPAO1 strain. Moreover, the introduction of p-*PA2826* to the Δ*PA2826-ospR* strain led to increased resistance to H_2O_2 and increased sensitivity to paraquat when compared with the Δ*PA2826-ospR* strain harbouring pAK1900 (Fig. S4). These results showed that OspR contributes to the oxidative stress response of *P. aeruginosa* and this process is mediated through PA2826.

PA2826 encodes a glutathione peroxidase (GPx) that catalyses the reduction of hydroperoxides, including H_2O_2, by using glutathione, and functions to protect bacterial cells from oxidative damage. To examine this role, we measured the redox state of the GSH/glutathione disulphide (GSSG) couple in MPAO1/pAK1900, Δ*ospR*/pAK1900, Δ*ospR*/p-*ospR* and MPAO1/p-*PA2826* strains, respectively, when bacteria were grown to an early stationary phase (OD_{600} = 2.2–2.3). As a result, Δ*ospR*/p-*ospR* displayed a similar GSH/GSSG ratio as that of wild-type MPAO1/pAK1900. However, both Δ*ospR*/pAK1900 and MPAO1/p-*PA2826* exhibited a 1.7-fold and 5.5-fold higher intracellular GSH/GSSG ratio compared with that of the wild-type strain.

OspR physically binds to the promoter of ospR-PA2826

Genetic evidence indicates that OspR regulates the expression of *PA2826* (Fig. 1A). In order to probe the putative OspR binding site, we examined the *PA2827–PA2826* intergenic region. An AT-rich inverted repeat sequence, 5′-ttcaatcaagttgtgtgcAATTgAATTgcgagctactt tattt-3′ (B2, uppercase letters indicate the conserved inverted repeat), was found (Fig. 2A). It is well known that the MarR family proteins specifically bind palindromic or pseudopalindromic sites using conserved winged helix fold (Wilkinson and Grove, 2006;Newberry *et al*., 2007). The inverted repeat overlaps the core promoter elements for *PA2826* (Fig. 2A). The direct interaction of OspR with *PA2826* promoter was tested by gel mobility shift assay using purified 6His-OspR and a 43 bp DNA fragment (B2) containing the putative OspR binding site. As shown in Fig. 2B, OspR bound to the 43 bp *PA2826* promoter sequence in the presence of non-specific salmon sperm DNA, while failed to shift a 43 bp DNA fragment (B1: 5′-agagcgacgacccgtctttggttcgggtcgtttttcattcaat-3′, Fig. 2A) of the *Rho*-independent terminator sequence as a control (data not shown).

Oxidation regulation of ospR *and* PA2826

We have shown that OspR contributes to bacterial oxidative stress response through regulating the expression of *PA2826*. To further assess whether *ospR* responds to oxidative stress, we treated *P. aeruginosa* MPAO1 (log phase, OD_{600} = 1.2–1.5) with different amounts of H_2O_2 for 10 min. Subsequently, the bacterial cells were harvested and the total RNAs were isolated. Northern blot analysis showed elevated transcripts of *PA2826* and *ospR* when the wide-type bacterial cells were treated with H_2O_2 as compared with the untreated bacteria (Fig. 2C). The transcription enhancements of *PA2826* and *ospR* are positively correlated with the concentrations of H_2O_2 applied to the bacterium. Increased transcriptions of *PA2826* mRNA were also observed when *P. aeruginosa* MPAO1 was treated with paraquat. As shown in Fig. 2D, the expression of *PA2826* is significantly induced by 0.05 μM paraquat in Luria–Bertani (LB) medium; however, unlike the situation with H_2O_2, higher concentrations of paraquat failed to further induce the expression of *PA2826*. We currently do not have an explanation for this observation with the paraquat treatment. Since increased transcription of *PA2826* is also detected in the Δ*ospR* strain (Fig. 1A), it is reasonable to suggest that OspR represses

PA2826-ospR, and oxidation with H_2O_2 leads to dissociation of OspR from the promoter DNA and de-repression of *PA2826* in *P. aeruginosa*. To confirm this hypothesis, we performed gel mobility shift assay with 6His-OspR and the same 43 bp promoter DNA fragment (B2) used in Fig. 2B. Addition of H_2O_2 or cumene hydroperoxide (CHP) led to dissociation of OspR from the DNA (Fig. 2E). In addition, the binding of OspR to the promoter DNA could be restored by the addition of excess reducing agent (DTT) (Fig. 2E).

OspR *affects pigment production in* P. aeruginosa

Unexpectedly, the introduction of p-*ospR* into Δ*ospR* in the complementary experiments described above also led to the production of dark red, water-soluble pigments when bacterial cells were grown on the LB agar plate (Fig. 3A). We were intrigued by this observation which suggests that OspR may have a broader role of regulation. We introduced p-*ospR* into the wild-type MPAO1 strain (MPAO1/p-*ospR*). MPAO1/p-*ospR* displayed the same dark red pigmentation on the LB agar plate (Fig. 3B) as observed with Δ*ospR*/p-*ospR*. This phenotype is independent of *PA2826* as MPAO1/*p-PA2826* exhibits the wild-type pigmentation (Fig. 3B), and Δ*PA2826-ospR*/pAK1900 and Δ*PA2826-ospR*/p-*PA2826* show the same normal pigmentation as MPAO1/pAK1900 while Δ*PA2826-ospR*/p-*ospR* displays the dark red pigment like the strain with constitutive expression of *ospR* when grown on the LB plate (Fig. S5A).

A previous study with mutation of two *P. aeruginosa* phenazine-modifying genes, *phzM* and *phzS*, led to accumulation of yellow and dark red phenazines (Mavrodi *et al*., 2001). To examine whether *ospR* affects the expression of these two *P. aeruginosa* phenazine-modifying genes, we performed the Northern blot analysis of mRNAs of *phzM* and *phzS*. As shown in Fig. 3C, the constitutive expression of *ospR* severely reduces the expression of both *phzM* and *phzS* in the MPAO1 strain. Consistent with this, the mRNA levels of *phzM* and *phzS* (Fig. 3D) were increased in the Δ*ospR* strain while further reduced in the Δ*ospR* strain complemented with p-*ospR* as compared with the wild-type strain (Fig. 3D).

Overexpression of OspR affects P. aeruginosa β-*lactam resistance*

The OhrR/MgrA family proteins are known to control oxidative stress response, bacterial virulence and antibiotic resistance (Fuangthong *et al*., 2001; Sukchawalit *et al*., 2001; Chen *et al*., 2006; 2008b). To assess whether OspR contributes to drug resistance in *P. aeruginosa*, we examined bacterial growth on TSA plates supplied with chloramphenicol, ciprofloxacin and cefotaxime respectively. Results showed that Δ*ospR* did not exhibit noticeable difference from the wild-type strain (data not shown) while MPAO1/p-*osp*R displayed higher resistance to cefotaxime (Fig. 3E) and ceftazidime (data not shown) when compared with the wild-type strain. This cefotaxime resistance phenotype is not mediated through *PA2826* as Δ*PA2826-ospR/p-ospR* showed the same resistance to cefotaxime as MPAO1/p-*ospR* (data not shown), and Δ*PA2826-ospR*/p-*ospR* displays enhanced resistance to cefotaxime when compared with Δ*PA2826-ospR*/pAK1900 (Fig. S5B).

Cys-24 as a key functional residue in OspR

OspR harbours two cysteine residues, Cys-24 and Cys-134 (Fig. S1). To investigate the contributions of these two residues to the function of OspR, each residue was mutated to serine. The *ospR* C24S mutant (in pAK1900, p-$ospR_{C24S}$), *ospR* C134S mutant (in pAK1900, p-$ospRC_{134S}$) and *ospR* C24S/C134S double mutant (in pAK1900, p-$ospR_{C24SC134S}$) were prepared and introduced into the wild-type MPAO1 strain respectively. The introduction of p-$ospR_{C24S}$, p-$ospR_{C134S}$ or p-$ospR_{C24SC134S}$ into the wild-type MPAO1 strain led to the production of the dark red, water-soluble pigments, a phenotype similar to that observed in MPAO1/p-*ospR* when bacteria were grown on LB agar plate (Fig. S6). In liquid culture, the MPAO1/p-*ospR* strain displayed the usual blue-green pigmentation (Fig. 4A). However, both

MPAO1/p-*ospR*$_{C24S}$ and MPAO1/p-*ospR*$_{C24SC134S}$ strains exhibited dark red pigmentation, while a yellow-green pigmentation was observed for the MPAO1/p-*ospR*$_{C134S}$ strain grown in LB liquid (Fig. 4A). This observation indicates that Cys-24 plays the key role in the regulatory function of OspR. We further examined β-lactam resistance of the two cysteine mutant strains. As shown in Fig. 4B, both OspR C24S mutant and OspR C24SC134S mutant strains showed enhanced β-lactam resistance but OspR C134S mutant showed normal sensitivity to β-lactam as the control strain (MPAO1/p-*ospR*).

To further investigate the role of Cys-24 in the regulatory function of OspR, we introduced p-*ospR*$_{C24S}$ into the *ospR* mutant strain and performed Northern blot analysis of transcripts of *PA2826* and *PA2850* (*ohr*). As shown in Fig. 4C, elevated transcriptions of *PA2826* and *ohr* were observed when the wide-type bacterial cells were treated with H_2O_2 as compared with the untreated bacteria, and this observation is consistent with previous transcriptional profiling result (Palma *et al*., 2004). There was no significant increase of the mRNA level of *PA2826* in the Δ*ospR*/p-*ospR* strain treated with H_2O_2 as compared with the untreated bacteria; however, a decrease of the mRNA level of *PA2826* was observed in the Δ*ospR*/p-*ospR*$_{C24S}$ strain treated with H_2O_2 compared with that of the untreated bacteria (Fig. 4D). Interestingly, an increase of *ohr* transcription was observed in the Δ*ospR*/p-*ospR*$_{C24S}$ strain treated with H_2O_2, but to a much less extent as compared with those of the Δ*ospR*/p-*ospR* and the wild-type strains treated with H_2O_2 (Fig. 4D). Thus, OspR also affects *ohr* and Cys-24 plays a major role in the regulatory function of OspR.

Next, we purified 6His-OspR C24S mutant protein and tested its interaction with the *PA2826* promoter (43 bpDNA fragment, B2) using gel mobility shift assay. This mutant protein binds the promoter DNA tighter than the wild-type OspR (Fig. 5A). Importantly, treatment with either H_2O_2 or CHP failed to dissociate OspR$_{C24S}$ from the 43 bp DNA fragment (B2) (Fig. 5B), indicating that Cys-24 is the key residue involved in the oxidant sensing. Not surprisingly, Cys-24 is the conserved cysteine used for oxidant sensing in MgrA, SarZ and OhrR (Fuangthong *et al*., 2001;Sukchawalit *et al*., 2001;Chen *et al*., 2006;2008a). Residues that form the hydrogen-bonding network (Tyr) with the redox-active cysteine and the nearby hydrophobic pocket (Tyr/Phe) are also conserved in OspR (Fig. S1).

Cysteine oxidation in OspR

Our next step was to confirm the redox activity of Cys-24 in OspR. We performed experiments to detect the potential formation of Cys-24-SOH in the wild-type OspR following CHP treatment by using NBD chloride (Chen *et al*., 2006; 2008a; Panmanee *et al*., 2006). However, this assay failed to yield an R-S(O)-NBD derivative in the CHP-oxidized OspR (data not shown). We suspected that this could be due to a rapid reaction of the generated sulphenic acid intermediate (R-SOH) with the second Cys residue, Cys-134, in OspR. We used non-reducing sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel to investigate whether intermonomer disulphides are formed in the oxidized OspR and OspR$_{C24S}$ *in vitro* (Panmanee *et al*., 2006; Chen *et al*., 2008a). Indeed, upon CHP treatment, OspR was oxidized to give a covalent dimer (Fig. 5C). Treatment with DTT prior to electrophoresis converted the oxidized protein back to its monomeric form, indicating a disulphide linkage between the two monomers in the oxidized OspR (Fig. 5C). We note that two distinct bands appeared in the size range corresponding to dimeric OspR (Fig. 5C). Formation of the lower dimeric protein band was dependent on the addition of the oxidant and the presence of Cys-24, while formation of the upper band did not require the presence of Cys-24 (Fig. 5C). The upper dimeric protein band could be a single-disulphide cross-link between two Cys-134 residues in the two monomers, whereas the lower dimeric band is doubly cross-linked OspR between Cys-24 from one monomer and Cys-134 from the other monomer (Soonsanga *et al*., 2008). To test if Cys-24-SOH is produced upon oxidation and subsequently trapped by Cys-134, the NBD chloride

trapping experiment was repeated using CHP-oxidized $OspR_{C134S}$. The UV-visible absorbance spectrum of NBD-labelled, oxidized $OspR_{C134S}$ exhibited a maximal absorbance at 347 nm, indicating formation of a sulphenic acid species in the oxidized $OspR_{C134S}$ protein (Fig. 5D).

OspR binds the PA2009–PA2010 *intergenic region and the promoter region of* PA1897

Six additional potential OspR binding targets (Table 1), corresponding to the promoter regions of 10 genes, were identified by a search in the *P. aeruginosa* genome sequence for the putative conserved OspR binding motif (AATTnAATT) located upstream (−1 bp to −400 bp) of the coding region by using RAST (http://rsat.ulb.ac.be/rsat/index.html). Interestingly, the promoter region of *PA1897* and the *hmgA*(*PA2009*)–*PA2010* intergenic region were found as a potential binding site for OspR (Table 1 and Fig. S7). While *PA1897* is a quorum-sensing-regulated gene, *hmgA* encodes an enzyme homogentisate-1,2-dioxygenase involved in tyrosine metabolism. Mutation of *hmgA* yielded strains with the hyperproduction of a dark-brown pyomelanin pigment in various *Pseudomonas* species including *P. aeruginosa*, *P. chlororaphis* O6 and *P. putida* (Arias-Barrau *et al*., 2004;Kang *et al*., 2008;Rodríguez-Rojas *et al*., 2009). An adjacent but oppositely transcribed gene *PA2010* (*hmgR*) was proposed to encode a regulator that induces *hmgA* expression when homogentisate is present (Arias-Barrau *et al*., 2004). We tested whether OspR binds to these two promoter regions using gel mobility shift assays. As shown in Fig. 6A, OspR could shift the 70 bp *hmgA*(*PA2009*)–*PA2010* intergenic region sequence (*hmgA*-p: 5′-tggcacgctggctgcgttatt tttatcgtAATTcAATTacgcataacgtaatttgagtggaaggcagcgt-3′) (uppercase letters indicate the conserved inverted repeat), and the 70 bp *PA1897* promoter sequence (*PA1897*-p: 5′-ggcaggttgtccctgccgggctgtgacAATTtAATTc gaccaggcatttcattgtccgtgccgattttca-3′) (uppercase letters indicate the conserved inverted repeat), respectively, while OspR failed to shift a 43 bp control DNA fragment (B2). To test whether the AATTnAATT direct repeat is required for the binding of OspR to the B2 DNA fragment, we performed the gel shift assay using a 34 bp B2d DNA fragment (B2 lacking AATTnAATT, 5′-ttcaatcaagttgtgtg cgcgagctactttattt-3′) and B2 DNA fragment as a control. As shown in Fig. 6B, OspR failed to bind to B2d DNA lacking the AT-rich repeat. Interestingly, the AT-rich inverted repeat sequence, 5′-AATTnAATT-3′, is similar to the putative OhrR box sequence thought to be involved in the binding of OhrR to the target promoters in *B. subtilis* (Fuangthong *et al*., 2001), *X. campestris* (Sukchawalit *et al*., 2001) and *Agrobacterium tumefaciens* (Chuchue *et al*., 2006). The role of this AATTnAATT motif in regulation awaits further study.

To further examine if *ospR* affects the expression of *PA1897*, *hmgA* and *PA2010*, we performed the Northern blot analysis of mRNAs of these three genes in MAPO1 (harbouring pAK1900), Δ*ospR* (harbouring pAK1900) and Δ*ospR*/p-*ospR* respectively. The result showed that deletion of *ospR* led to increased expression of *PA1897* while decreased expression of *hmgA* and *PA2010* as compared with those of the wild-type strain when bacteria were grown in early stationary phase (OD_{600} = 2.5). Complementation with p-*ospR* in Δ*ospR* restored the mRNA levels of *hmgA*, *PA2010* and *PA1897* to normal levels observed in the wild-type MPAO1 strain (Fig. 6C).

Virulence phenotypes in animal experiments

The *in vitro* results presented above suggest that *ospR* plays global roles in oxidative stress response, pigment production and antibiotic resistance. We tested whether *ospR* also impacts the virulence in a mouse model of acute pneumonia. C57BL/6 mice were infected intranasally with approximately 1×10^7 wild-type bacteria (MPAO1/pAK1900), *ospR* null mutant bacteria (Δ*ospR*/pAK1900) or *ospR* null mutant bacteria expressing *ospR* in pAK1900 (Δ*ospR*/p-*ospR*). Figure 7 shows the ratio of bacteria recovered from the lungs and spleens relative to the initial inoculum at 18 h post infection, with geometric means indicated for each group. In this

assay, the wild-type MPAO1/pAK1900 was recovered in numbers approximately at 0.008% and 0.00001% of initial inoculum dose from lungs and spleens respectively. In marked contrast, Δ*ospR*/pAK1900 bacteria were recovered in numbers equal to approximately 0.08% of the inoculum dose from lungs and 0.0005% of the inoculum dose from spleens. These differences achieve statistical significance. Further, bacteria of the complementary strain (Δ*ospR*/p-*ospR*) were recovered from lungs or spleens with a similar recovery ratio to that of the wild-type MPAO1/pAK1900 strain. These results indicate that *ospR* has an impact on the capacity for dissemination in this model. Mutation of *ospR* leads to an increased bacterial virulence.

Discussion

Reactive oxygen species were originally considered to be exclusively detrimental to cells; however, redox regulation involving ROS is now recognized as a vital component to cellular signalling and regulation (Scandalios, 1997; Imlay, 2008; Poole and Nelson, 2008). In this study, we identified a new gene, *ospR*, which encodes a MarR family protein in *P. aeruginosa*. OspR is a functional homologue of the bacterial OhrR/MgrA family oxidative stress sensing and regulatory proteins. OspR binds to the promoter of *PA2826*, which encodes a glutathione peroxidase, and represses the expression of *PA2826* and likely itself. Additional sites (*hmgA–PA2010* intergenic region and *PA1897* promoter region) in the *P. aeruginosa* genome may be recognized by OspR. OspR may bind to some of these sites and exert regulatory functions. It is still unclear if OspR can act as a direct transcriptional activator. A cysteine residue, Cys-24, is used by OspR to sense potential oxidative stress and regulates bacterial response. Oxidation of Cys-24 in OspR leads to the dissociation of the protein from promoter DNA. OspR is also involved in pigment production, impacts β-lactam resistance and affects dissemination during infection. Thus, it is a global regulator that controls multiple pathways in *P. aeruginosa*.

Orthologues of *ospR* are present in various *P. aeruginosa* and *P. fluorescens* strains while absent in some other *Pseudomonas* species such as *P. putida*, *P. syringae* and *P. entomophila L48*. This gene in *P. aeruginosa* and *P. fluorescens* may help to provide an optimal response to the altered redox environment for these bacteria. This response seems to be partially mediated through regulation of PA2826, a glutathione peroxidase. GPx is an enzyme that removes H_2O_2 with the oxidation of glutathione. Glutathione reductase recycles glutathione for further H_2O_2 removal (Miller and Britigan, 1997). Thus, it is not surprising that either de-repression of *PA2826* in Δ*ospR* or constitutive expression of *PA2826* in the wild-type MPAO1 increases the bacterial resistance to H_2O_2.

Unexpectedly, constitutive expression of *PA2826* led to a higher intracellular GSH/GSSG ratio and a reduction in bacterial paraquat resistance. The mechanism underlying these observations is currently unknown. Perhaps, the higher intracellular GSH/GSSG ratio is caused by a compensation pathway induced by overexpression of *PA2826*, as proposed in Fig. 8. It has been reported that a *P. aeruginosa zwf* mutant shows an increased sensitivity to paraquat and it is believed that the NADPH level is essential in defending against paraquat toxicity (Ma *et al*., 1998). The *zwf* gene encodes glucose-6-phosphate dehydrogenase (G6PDH), an enzyme that catalyses the conversion of glucose-6-phosphate to 6-phosphogluconate while producing NADPH (Ma *et al*., 1998). Depletion of NADPH via glutathione reductase-catalysed production of GSH may explain the higher sensitivity of the Δ*ospR* strain towards paraquat. This hypothesis needs to be further tested experimentally in the future.

Distinct from OhrR, OspR plays multiple regulatory roles as a transcriptional regulator in addition to protection against oxidative stress, as evidenced by the effects of *ospR* on pigment production and β-lactam resistance in a *PA2826*-independent manner. OspR is not required for β-lactam resistance in *P. aeruginosa*, considering that *ospR* mutant showed no increased

sensitivity to cefotaxime or ceftazidime compared with the wild-type MPAO1 strain. However, enhanced expression or activation of OspR may contribute to the increased β-lactam resistance in *P. aeruginosa*. How OspR impacts β-lactam resistance is unclear and awaits further study. *PA1874*, a potential OspR-regulated gene (Table 1), has been reported to associate with antibiotic resistance in *P. aeruginosa* (Zhang and Mah, 2008).

Pseudomonas species are well known to produce multiple-coloured phenazine pigments. These molecules can undergo redox cycling to produce toxic superoxide and H_2O_2, and play roles in bacterial virulence and redox balance (Price-Whelan *et al*., 2006). OspR affects the expression of two phenazine-modifying genes, *phzM* and *phzS*, indicating that *OspR* impacts on phenazine biosynthesis or modification (Fig. 3). Interestingly, the MPAO1/p-*ospR* strains exhibit the dark red pigmentation on agar plates while it shows a normal blue-green pigmentation in liquid culture (Fig 3 and Fig 4). The pathways underlying this observation are still unknown. *P. aeruginosa* produces several pigmented chemicals in addition to phenazine and the final colour is the combination of these pigments. We found that OspR binds to the promoter region of *hmgA* and affected its expression. The *hmgA* gene has recently been shown to be involved in pyomelanin pigment (dark-brown) production (Rodríguez-Rojas *et al*., 2009). This pathway could contribute to the observed pigment phenotypes.

The regulations of pigment production and quorum-sensing-regulated genes by OspR are unique observations for the OhrR/MgrA family redox-active regulatory proteins. Aside from regulating the expression of *phzM* and *phzS* (Fig. 3), two well-known quorum-sensing-regulated genes (Wagner *et al*., 2006), OspR also binds to the promoter of *PA1897* and exerts a regulatory function (Fig. 6). *PA1897* is a quorum-sensing-regulated gene controlled by QscR, which is a modulator of quorum-sensing signal synthesis and virulence (Chugani *et al*., 2001;Lee *et al*., 2006). It has been known that *P. aeruginosa* quorum sensing controls expression of catalase and superoxide dismutase genes (Hassett *et al*., 1999). Our results reveal additional links between oxidative response and quorum sensing through OspR in *P. aeruginosa*.

The chronic lung infection in cystic fibrosis (CF) patients is a state of chronic oxidative stress (Wood *et al*., 2001; Lagrange-Puget *et al*., 2004). In CF *P. aeruginosa* infections, bacteria routinely reach very high densities within the respiratory secretions [10^8 to 10^{10} colony-forming units (cfu) ml^{-1}] (Hoiby, 1998) and their infections are thought to involve co-ordinated bacterial activities facilitated by quorum sensing systems (Wagner and Iglewski, 2008; Willcox *et al*., 2008; Winstanley and Fothergill, 2009). The cross-talk between oxidative stress and quorum sensing system revealed in this study may co-ordinate various pathways in *P. aeruginosa* to cope with changes in the host environment.

There are two subfamilies of OhrR type redox-active regulatory proteins (Panmanee *et al*., 2006; Soonsanga *et al*., 2008). The 1-Cys type OhrR proteins sense peroxides by formation of a sulphenic acid intermediate that may further react with intracellular small molecule thiols to give a mixed disulphide. The 2-Cys type proteins sense peroxides by forming intermonomer disulphides. OspR belongs to the 2-Cys class as indicated in Fig. 5C. Cys-24 is a key residue involved in pigment production, drug resistance, and expression of oxidative stress-related genes such as *PA2826* and *ohr* in OspR (Fig. 4). Our data indicate that Cys-24 is likely oxidized first, and the resulting sulphenic intermediate is trapped by Cys-134 to form an intermonomer disulphide. Unexpectedly, although H_2O_2 induced the transcription of *PA2826* in the wild-type MPAO1 strain, it failed to induce the expression of *PA2826* in the complementation strain (Δ*ospR*/p-*ospR*) in our study (Fig. 4D). It is likely that the constitutive expression of OspR in Δ*ospR*/p-*ospR* yielded a large excess of OspR which compromised its redox-sensitive regulation of *PA2826* when bacteria were treated with H_2O_2.

Lastly, the Δ*ospR* strain shows enhanced dissemination in a murine model of acute pneumonia (Fig. 7). The de-repression of *PA2826* (Fig. 1), and the enhanced expression of *phzS* and *phzM* (Fig. 3) may contribute to increased bacterial virulence observed for this strain, considering that de-repression of *PA2826* leads to increased resistance to H_2O_2 (Fig. 2) while *phzS* and *phzM* are required for full virulence of *P. aeruginosa* in murine lungs (Lau *et al*., 2004a,b). In addition, the downregulation of *hmgA* in Δ*ospR* (Fig. 6C) may help bacteria adapt to immune systems since disruption of *hmgA* leads to higher resistance to oxidative stress and increased persistence in chronic lung infections (Rodríguez-Rojas *et al*., 2009). All of these pathways intersect at OspR, showing a connection between oxidative stress response and dissemination in pathogenic *P. aeruginosa.* These results also suggest that exposure to certain levels of oxidative stress may switch on defensive pathways in *P. aeruginosa*, thus rendering the bacterium more resistant to killing by immune cells. Similarly, it has been shown that nitric oxide-mediated activation of bacterial defence is important for the *in vivo* virulence of *Bacillus anthracis* (Shatalin *et al*., 2008). An interesting avenue for future research therefore would be to identify genes targeted by OspR in a genome scale, which should help to shed light on the role of oxidative stress response in *P. aeruginosa* physiology and pathogenesis.

Experimental procedures

Bacterial strains and growth conditions

Pseudomonas aeruginosa and *Escherichia coli* strains were maintained in LB medium. All *P. aeruginosa* and *E. coli* strains used in this study are listed in Table 2. For plasmid maintenance in *E. coli*, the medium was supplemented with 100 μg ml^{-1} ampicillin, 10 μg ml^{-1} tetracycline or 15 μg ml^{-1} gentamicin. For marker selection in *P. aeruginosa*, 100 μg ml^{-1} tetracycline or 30 μg ml^{-1} gentamicin was used, as appropriate.

Nucleotide sequences (5–3′) of the primers

FD2825downF: GTTTCTAGACAACGAGATGTTCGACCT GC; FD2825downR: TTTCTGCAGCCTGGAGTACAAGCG TCTGG; FD2825upF: TTTGGATCCGGGTTGCGTTACTG CATCA; FD2825upR: TTTTCTAGAAGCAGCAGCGATT CTTCG; Tet-XbaI-F: ATTTCTAGATTTCAGTGCAATTTAT CT; Tet-XbaI-R: TTTTCTAGAGGACGCGATGGATATGTT CT; D26-25UpF: CTCGAATTCCCACCTGCTTCTGCTGGTA; D26-25UpR: TCCTCTAGAAATCGCTCATGGCTTGTCTT; D26-25DownF: TCCTCTAGACTCAACGAGATGTTCGACC TG; D26-25DownR: TCGAAGCTTCCTGGAGTACAAGCGT CTGG; PA2825FCF: TTAAGCTTGCATCAAGTGGAACT TCACC; PA2825FCR: TTGGATCCACTACCTGGCCAAGC CTTTC; FO2826F: TGCAAGCTTTATTTCGAGACCCAC CCTCA; FO2826R: CGGGGATCCGGCGTACAGCTTGAAA CACA; PA2827FNF: GTTGACCGAAGAGCAGTTCC; PA2827FNR: GTGGCTGAAGTCGTCCAGTT; PA2826FNF: ATCAAGGGCGAACAGAAGAC; PA2826FNR: GAAGCT CACCCCGTAGTTCA; PA2824FNF: CTTCGTAGCCGTC CATCACT; PA2824FNR: CTACGGCAGTTTCCTCGAAC; *phzM*-FNF: CTGCTGCGCGTAATTTGATA; *phzM*-FNR: CAA CAGGCTGGAAAGGTTGT; *phzS*-FNF: GGAAAGCAG CAGCGAGATAC; *phzS*-FNR: CGGGTACTGCAGGATC AACT; C24SF: GCTCGACAACCAGCTGAGTTTCAAGCTG TACGC; C24SR: GCGTACAGCTTGAAACTCAGCTGGT TGTCGAGC; hmgAFNF: GAGGTCAGCACGGTGAAGAT; hmgAFNR: CTACCAGTACCTGGCCAACC; PA2010FNF: CGCCTCCACCAATCATTACT; PA2010FNR: CAACTGAT AGCCCGAGTCGT; PA1897FNF: AGATCGGGAAGTCG CTGTAG; PA1897FNR:

CGGGTGATCTTCCTCAACAT; Pa2825C2toSF: TGCGCCAGCAGCTGATCTCCAGCACCG GTTTCGACCT; Pa2825C2toSR: AGGTCGAAACCGGT GCTGGAGATCAGCTGCTGGCGCA; PA2850FNF: CTCGA CGTGAAACTCAGCAC; PA2850FNR: GTTGGAGTAGGG GCAGACCT; PA2825F-NdeI: TTGGACACATATGATGAG CACCCGGGGAAAAGT; PA2825R-XhoI: CACACTCGA GCTAGCCTCCTACCACCAGACGGA.

Construction of P. aeruginosa ΔospR *and* ΔPA2826-ospR *mutants*

For gene replacement, a *sac*B-based strategy (Schweizer and Hoang, 1995) was employed. To construct the *ospR* null mutant (Δ*ospR*), polymerase chain reactions (PCRs) were performed to amplify sequences upstream (706 bp) and downstream (764 bp) of the intended deletion. The upstream fragment was amplified from MPAO1 genomic DNA using primers FD2825upF (with BamHI site) and FD2825upR (with XbaI site), while the downstream fragment was amplified with primers, FD2825downF (with XbaI site) and FD2825downR (with PstI site, see in *Experimental procedures*). The two PCR products were digested with BamHI–XbaI or XbaI–PstI, as appropriate, and then cloned into BamHI/PstI-digested gene replacement vector pJQ200mp18 via a three-piece ligation, which yielded pJQ200mp18::2825UD. A tetracycline resistance cassette was amplified from EZ-Tn*5* <TET-1> (EPICENTRE® Biotechnologies) with primers, Tet-XbaI-F and Tet-XbaI-R (see in *Experimental procedures*). The PCR product was digested with XbaI and cloned into XbaI-digested pJQ200mp18::2825UD. The resultant plasmid, pJQ200mp18::2825UTD, was electroporated into MPAO1 with selection for tetracycline resistance. Colonies were screened for gentamicin sensitivity and loss of sucrose (5%) sensitivity, which typically indicates a double-cross-over event and thus of gene replacement occurring. The Δ*ospR* strain was further confirmed by PCR and Southern blot analysis.

The Δ*PA2826-ospR* strain was constructed by a similar strategy using the suicide vector pEX18Ap::26-25UD. Briefly, the 972 bp fragments of the upstream region of *PA2826* gene were amplified using primers D26-25UpF (with EcoRI site) and D26-25UpR (with XbaI site). The primers D26-25DownF (with XbaI site) and D26-25DownR (with HindIII site) were used for amplification of 765 bp of *PA2825* downstream region. The two PCR products were digested with EcoRI–XbaI or XbaI– HindIII, as appropriate, and then cloned into EcoRI/HindIII-digested gene replacement vector pEX18Ap via a three-piece ligation, yielding pEX18Ap::26-25UD. A 1.8 kb gentamicin resistance cassette was cut from pPS858 with XbaI and then cloned into pEX18Ap::26-25UD, yielding pEX18Ap::26-25UD.

Plasmid construction for constitutive expression of ospR *and* PA2826

In order to construct the plasmid for constitutive expression of *ospR*, the following were amplified using primers PA2825FCF (with HindIII site) and PA2825FCR (with BamHI site): a 705 bp PCR product covering 115 bp of the *ospR* upstream region, the *ospR* gene, and 98 bp downstream of *ospR* gene. The product was digested with HindIII and BamHI and ligated into PAK1900 in the same orientation as p*lac* to generate p-*ospR*. To construct the plasmid for constitutive expression of *PA2826*, a 605 bp PCR product covering 36 bp of the *PA2826* upstream region, the *PA2826* gene, and 84 bp downstream of *PA2826* was amplified using primers FO2826F (with HindIII site) and FO2826R (with BamHI site) and then cloned into PAK1900, yielding p-PA2826. The three mutations, p-$OspR_{C24S}$, p-$OspSPR_{C134S}$ and p-$OspR_{C24SC134S}$, were obtained by using QuikChange II site-directed mutagenesis kit (Stratagene). Primer pairs C24SF/C24SR and Pa2825C2toSF/Pa2825C2toSR were used for generating C24S and C124S mutation respectively. All the constructs were sequenced to ensure that no unwanted mutations resulted.

RNA isolation and Northern blotting

Total RNA was isolated using a Qiagen RNeasy kit according to the manufacturer's recommendations. RNA concentration and purity were determined by absorbency at 260 and 280 nm. Northern blotting was performed following previously reported procedures (Chen *et al*., 2008a).

Construction, expression and purification of 6His-OspR, 6His-OspR$_{C24S}$ and 6His-OspR$_{C134S}$

OspR was cloned into pET28a with a thrombin-cleavable N-terminal His-tag and expressed in *E. coli* strain BL21 (DE3) (Novagen). Two sets of primers were used to amplify the *OspR* gene from *P. aeruginosa* MPAO1 chromosomal DNA: PA2825-NdeI and PA2825-XhoI. The amplified fragments were ligated into similarly cut pET28a (Novagen) to produce the plasmids pET28a-his-*OspR*. *OspR*$_{C24S}$ and *ospR*$_{C134S}$ was amplified from p-*ospR*$_{C24S}$ and p-*ospR*$_{C134S}$ by using primer pair PA2825F-NdeI/PA2825R-XhoI and then cloned into pET28a, respectively, as described above. Clones were verified by DNA sequencing and transformed into BL21 (DE3) for expression.

The expression and purification procedures for OspR, OspR$_{C24S}$ and OspR$_{C134S}$ are described as follows. The strains were grown at 37°C overnight in 10 ml of LB medium containing 50 μg ml^{-1} kanamycin (LB$_{kan50}$). The next day, the cultures were transferred into 1 l of LB$_{kan50}$, incubated at 37°C until the OD$_{600}$ reached 0.6, after which IPTG (isopropyl-1-thio-β-D-galactopyranoside) was added to a final concentration of 1.0 mM.After 4 h incubation at 30°C, the cells were harvested by centrifugation and stored at −80°C. The cells were lysed at 4°C by sonication in lysis buffer [10 mM Tris (pH 7.4), 300 mM NaCl, 1 mM PSMF and 2 mM DTT]. Clarified cell lysate was loaded onto a HisTrap HP column (Amersham Biosciences), washed with Ni-NTA washing buffer and eluted with Ni-NTA elution buffer. The fractions containing OspR, OspR$_{C24S}$ or OspR$_{C134S}$ were concentrated and loaded onto a Superdex-200 gel filtration column with a running condition of 10 mM Tris (pH 7.4), 300 mM NaCl and 2 mM DTT.

Electrophoretic mobility shift assays

The electrophoretic mobility shift experiments were performed by using [γ-^{32}P]-ATP labelled method. Duplex DNA (5′–3′ annealed to its complementary strand) containing various promoter regions was used for the assay. DNA fragments were labelled using [γ-^{32}P]-ATP (Perkin Elmer) and a T4 polynucleotide kinase. Unincorporated dATP were removed using illustra MicroSpin™ G-50 Columns (GE Healthcare). The electrophoretic mobility shift experiments were performed by adding 0.04 pmol of P^{32}-labelled duplex DNA to 24 μl of reaction buffer (10 mM HEPES at pH 8.0, 1 mM EDTA, 50 mM KCl, 0.05% Triton X-100, 10% glycerol, 10 μg ml^{-1} salmon sperm DNA). Reactions were placed on ice for 30 min before loading. Designated amounts of H_2O_2 or CHP were used as appropriate. Gels were run in 0.5× TBE at 85 V at room temperature. The gels were dried and subjected to autoradiography using the storage phosphor screen (Image Screen-K, Kodak) and the Molecular Imager PharosFX Plus System (Bio-Rad).

Oxidative and drug stress plates

LB agar plates were made with designated amounts of paraquat, H_2O_2 and cefotaximine. The overnight culture was diluted 100-fold in fresh LB medium. *P. aeruginosa* were grown to early stationary phase (OD$_{600}$ = 2.0) in LB broth, and 10-fold dilutions were made. Aliquots (10 μl) of the diluted cultures for each strain were spotted onto the solid media and grown at 37°C.

Glutathione (GSH)/glutathione disulphide (GSSG) measurements

The overnight culture was diluted 100-fold in fresh LB media and incubated at 37°C for 6 h until the culture reached OD_{600} = 2.2–2.3. The pellet from 10 ml of a cell culture was stored at −80°C and re-suspended in 0.25 ml of double-distilled water before used. GSH/GSSG ratio was measured by using GSH/GSSG Cuvette Assay Kit (Catalog # GT35, Oxford Biomedical Research).

Analysis of OspR oxidation in vitro *by SDS-PAGE and chemical characterization of sulphenic acid modification*

Purified His6-tagged OspR proteins were washed with the thiol-assay buffer (100 mM KH_2PO_4/K_2HPO_4, 200 mM NaCl and 1 mM EDTA at pH 7.0) four times to remove extra DTT using Microcon YM-10 (Amicon) ultrafiltration device. Protein samples (50 μM, monomer) were treated with four equivalents of CHP (200 μM) at room temperature for 10 min followed by washing with the thiol-assay buffer three times to generate the oxidized OspR. Disulphide bond formation was monitored by non-reducing SDS-PAGE. The $OspR_{C134S}$-SOH modification was confirmed by the NBD-Cl assay (Chen *et al*., 2006).

Mouse model of acute pneumonia

Mouse infections were carried out as described previously (Laskowski *et al*., 2004), using 8-week-old female C57BL/6 mice obtained from the National Cancer Institute and housed under specified pathogen-free conditions. All studies were approved by the Yale University Institutional Animal Care and Use Committee. Mice were lightly anaesthetized with isoflurane and intranasally infected with *c*. 2×10^7 cfu of each bacterial isolate; the actual inoculum titre for each group was determined by plating serial dilutions. Animals were sacrificed 18 h post infection. Lungs and spleens were aseptically removed and homogenized in PBS plus 0.1% Triton X-100 to obtain single-cell suspensions. Serial dilutions of each organ were plated on VBM (Vogel–Bonner minimal) agar plates. Bacterial burden per organ was calculated and is expressed as a ratio of the inoculum delivered per animal. Statistical analysis was performed using Prism software (GraphPad).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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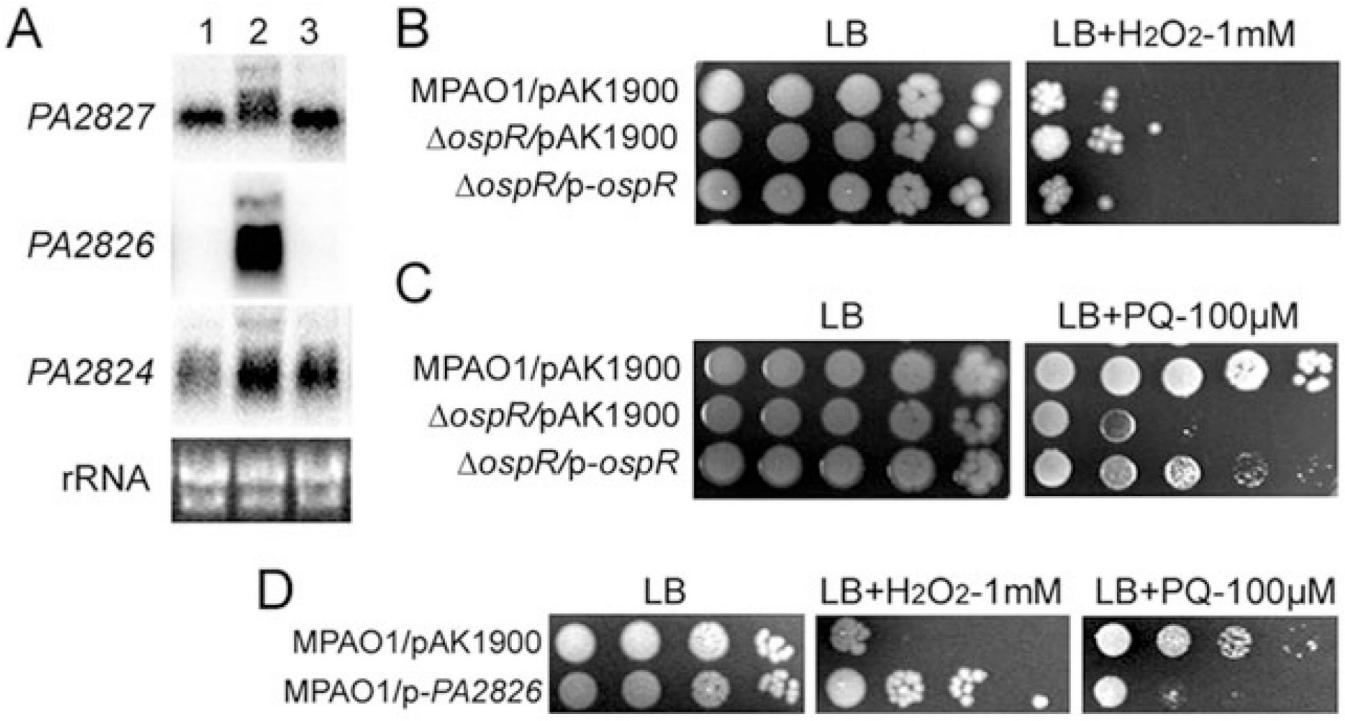

Fig. 1.

Northern blot analysis and phenotypes of *P. aeruginosa* strains under oxidative stresses.

A. Northern blot analysis of the transcripts of *PA2827 PA2826* and *PA2824*. Total cellular RNA samples were obtained at log phase (the overnight culture was diluted 100-fold in fresh media and incubated at 37°C for 2.5–3 h until the culture reached OD_{600} = 1.2–1.5). The coding region of each gene was polymerase chain reaction (PCR) amplified and radiolabelled with ^{32}P-dCTP as probes. All genes were tested with the same RNA samples. Five micrograms of total cellular RNA was used in each experiment with the ethidium bromide-stained gel picture of the loaded RNA sample shown below each lane. 1: MPAO1/pAK1900; 2: Δ*ospR*/pAK1900; 3: Δ*ospR*/p-*ospR*.

B and C. The *ospR* null mutant strains show increased resistance to H_2O_2(B) and sensitivity to paraquat (PQ) (C) compared with wild-type MPAO1 strains (harbouring pAK1900) and Δ*ospR*/p-*ospR* strains. *P. aeruginosa* strains were grown on LB agar plates with or without H_2O_2 (1 mM) at 37°C for 24 h (B). *P. aeruginosa* strains were grown on LB agar plates with or without paraquat (0.1 mM) at 37°C for 48 h (C).

D. The constitutive expression of *PA2826* enhances *P. aeruginosa* MPAO1 growth on LB agar plates containing H_2O_2 (1 mM) but reduces *P. aeruginosa* MPAO1 growth on LB agar plates supplied with paraquat (PQ) (0.1 mM). *P. aeruginosa* strains were grown at 37°C for 24 h. All experiments were repeated several times and similar results were obtained.

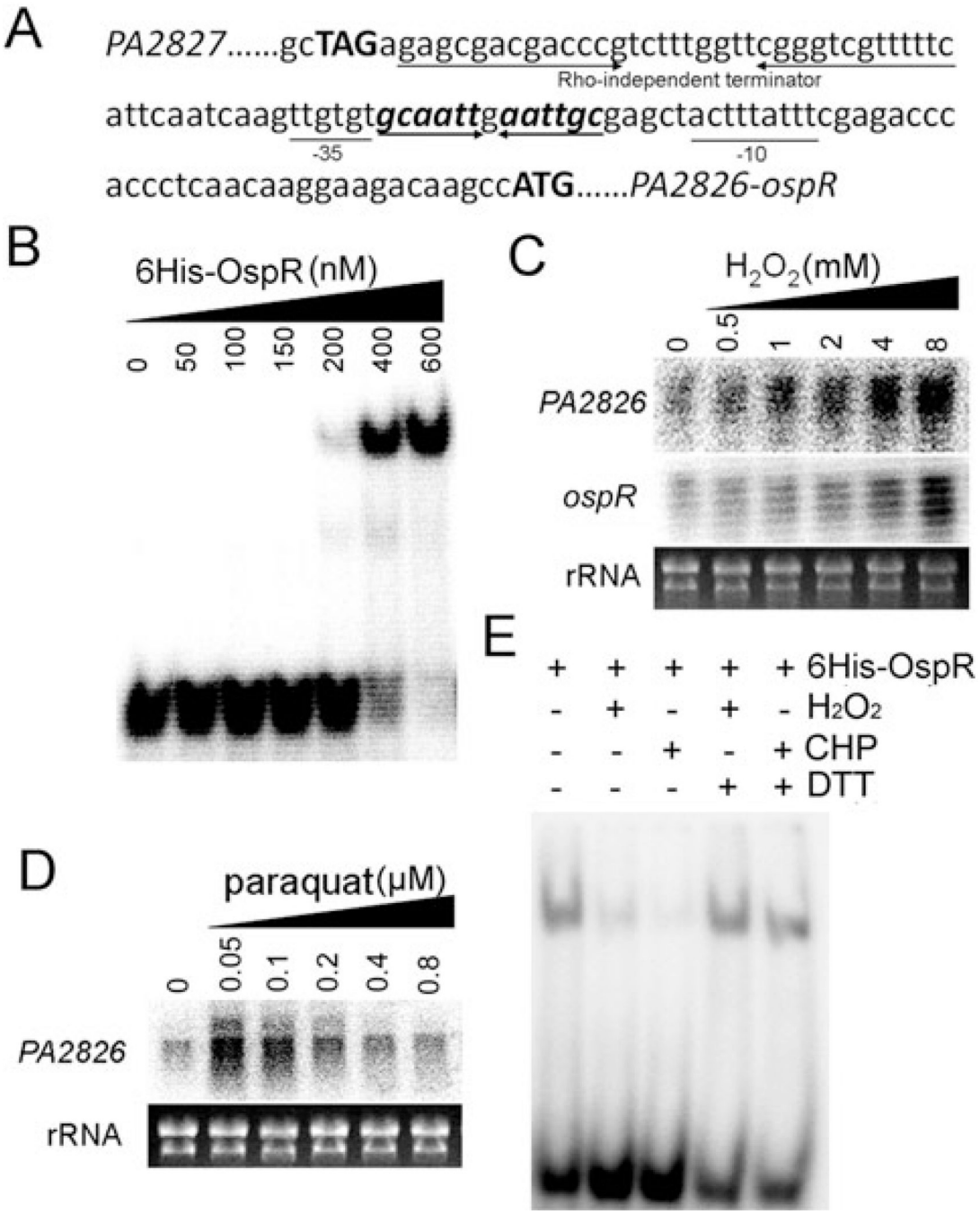

Fig. 2.

The proposed OspR binding site, gel shift assay and Northern blot analysis.

A. The *PA2826–PA2827* intergenic region. The putative −35 and −10 elements are underlined and arrows show the palindromic putative OspR-binding sequence. A putative Rho-independent terminator is also indicated.

B. A gel shift experiment showing binding of 6His-OspR to this promoter DNA.

C. Northern blots analysis of the transcripts of *ospR PA2826* in *P. aeruginosa* MPAO1 strains untreated and/or treated with H_2O_2.

D. Northern blots analysis of the transcripts of *PA2826* in *P. aeruginosa* MPAO1 strains untreated and/or treated with paraquat. The overnight culture was diluted 100-fold in fresh

media and incubated at 37°C for 2.5–3 h until the culture reached OD_{600} = 1.2–1.5 before treatment with H_2O_2 or paraquat.

E. Electrophoretic mobility shift assay showing the effect of oxidation on the DNA binding of purified OspR. A gel shift experiment showed binding of 6His-OspR to this promoter DNA. Addition of H_2O_2 (0.5 mM) or CHP (32 mM) led to dissociation of 6His-OspR from this promoter DNA. When indicated, 1 mM DTT was added after incubation of the protein with oxidants for 30 min, and the mixture was incubated for an additional 30 min at room temperature before samples were run on the gel; 300 nM 6His-OspR was used in each reaction.

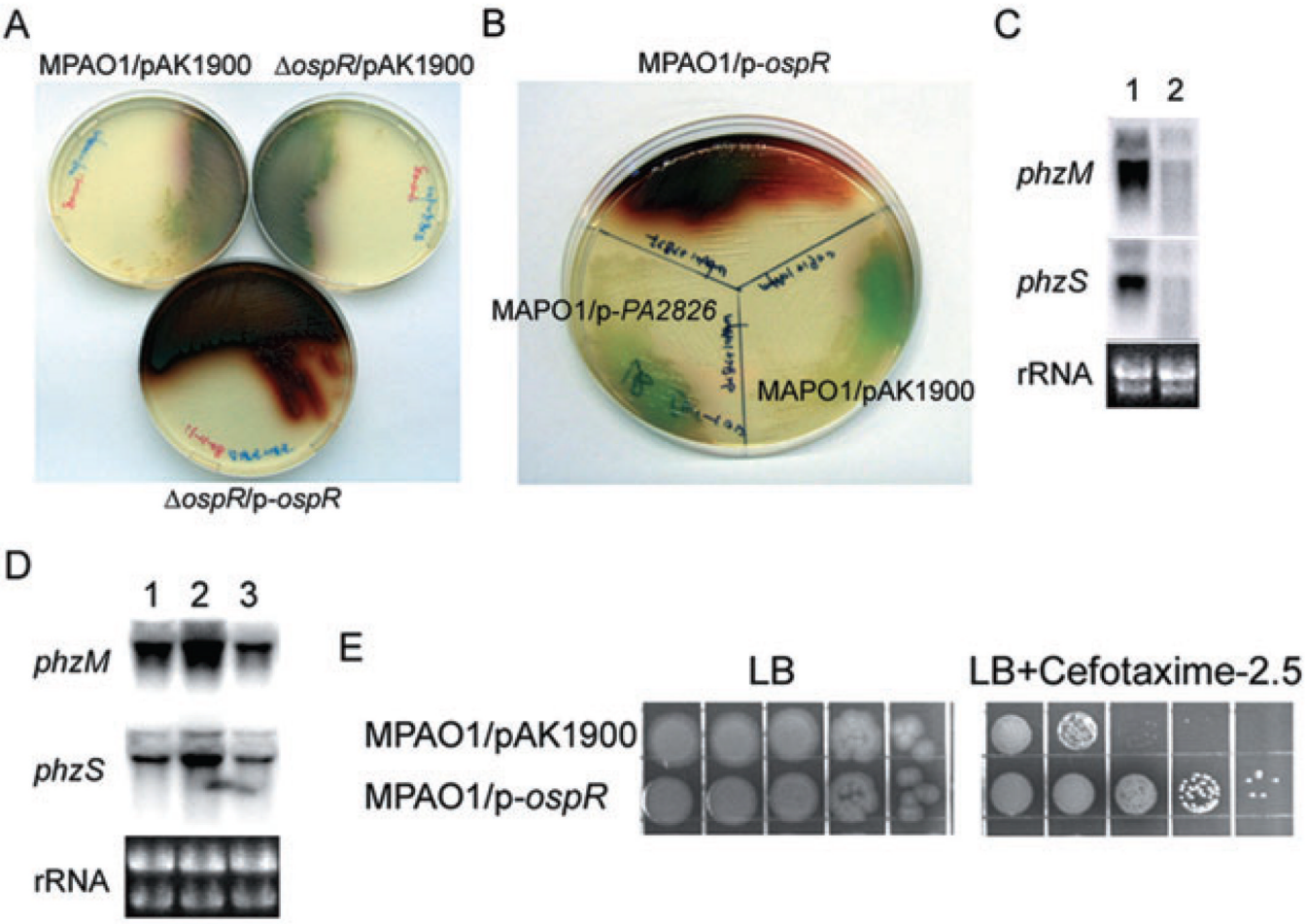

Fig. 3.

Constitutive expression of *ospR* in *P. aeruginosa*.

A. Phenotype of MPAO1/pAK1900 strains, Δ*ospR*/pAK1900 and Δ*ospR*/p-*ospR* strains grown on LB agar plate at 37°C for 24 h.

B. Phenotype of MPAO1/pAK1900 strains, MPAO1/p-*PA2826* and MPAO1/p-*ospR* strains grown on LB agar plates at 37°C for 20 h.

C. Northern blot of *phzS* and *phzM* showing that constitutive expression of *ospR* decreased the expression of these two phenazine-modifying genes. The overnight culture was diluted 1000-fold and 50 μl of dilution was plated on LB agar plate. Bacteria were harvested after incubating at 37°C for 24 h and then subjected to total RNA isolation. 1: MPAO1/pAK1900; 2: MPAO1/p-*ospR*.

D. Northern blot of *phzS* and *phzM*. The overnight culture was diluted 100-fold in fresh LB medium. Bacteria were harvested after incubating at 37°C for 9 h (OD_{600} = 2.5) and then subjected to total RNA isolation. 1: MPAO1/pAK1900; 2: Δ*ospR*/pAK1900; 3: Δ*ospR*/p-*ospR*.

E. The constitutive expression of *ospR* enhances growth of *P. aeruginosa* MPAO1 strain on LB agar plate containing cefotaxime (2.5 μg ml^{-1}). The plates were incubated at 37°C for 48 h. All experiments were repeated multiple times with similar results obtained.

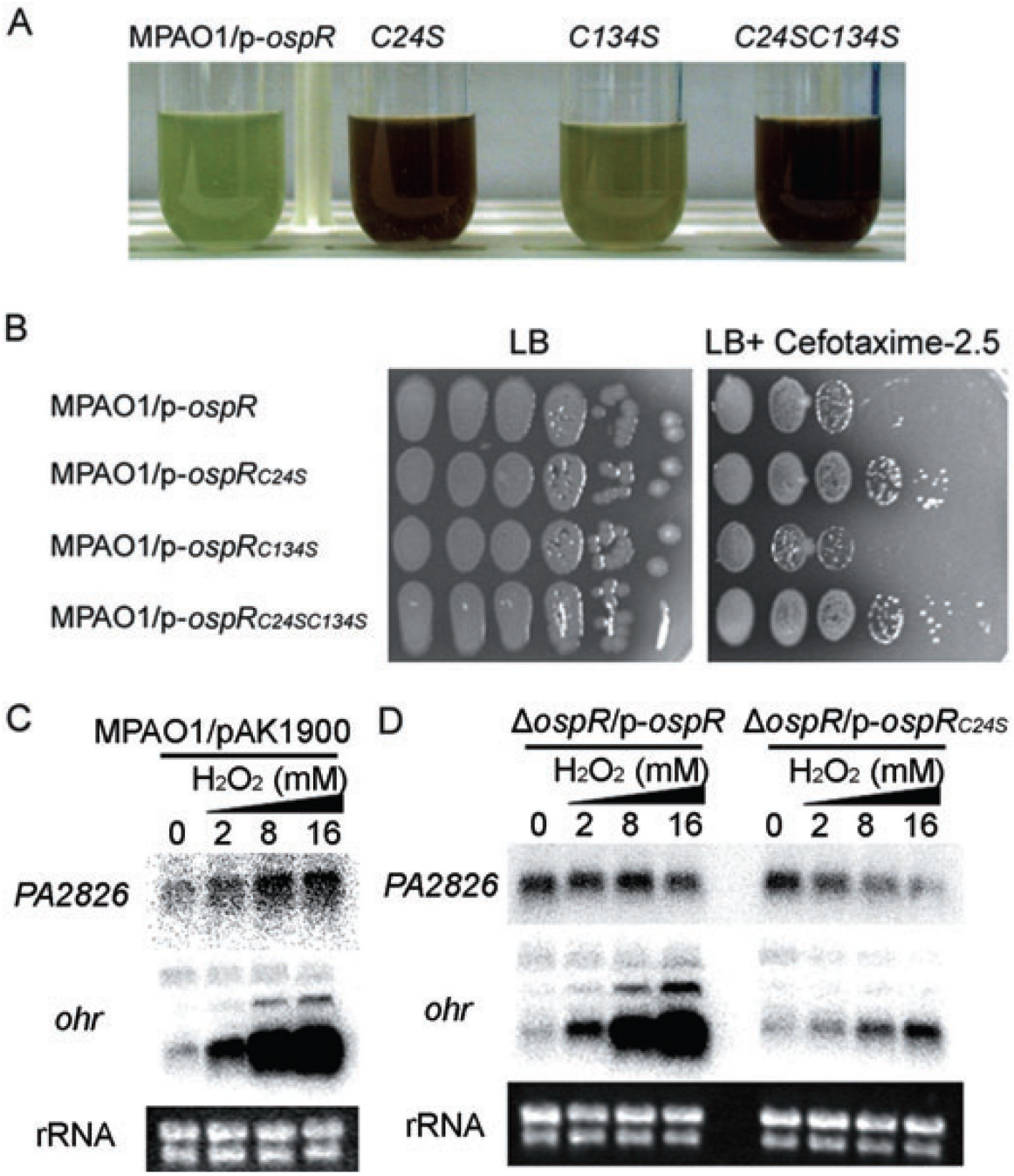

Fig. 4.

Phenotypes of *P. aeruginosa* strains and Northern blot assay.

A. The overnight culture was diluted 100-fold in fresh LB medium and then incubated at 37° C for 16 h with shaking at 250 r.p.m. *C24S*: MPAO1/p-*ospR*$_{C24S}$; *C134S*: MPAO1/p-*ospR*$_{C134S}$; *C24SC134S*: MPAO1/p-*ospR*$_{C24SC134S}$.

B. *P. aeruginosa* MPAO1 strain grown on LB agar plate containing cefotaxime (2.5 μg ml^{-1}) incubated at 37°C for 48 h.

C and D. Northern blot analysis of the transcripts of *PA2826* and *ohr* (*PA2850*) in the *P. aeruginosa* MPAO1, Δ*ospR*/p-*ospR* and Δ*ospR*/p-*ospR*$_{C24S}$ strains treated with or without

H_2O_2. The overnight culture was diluted 100-fold in fresh media and incubated at 37°C for 2.5–3 h until the culture reached OD_{600} = 1.2–1.5 before treatment with H_2O_2 for 10 min.

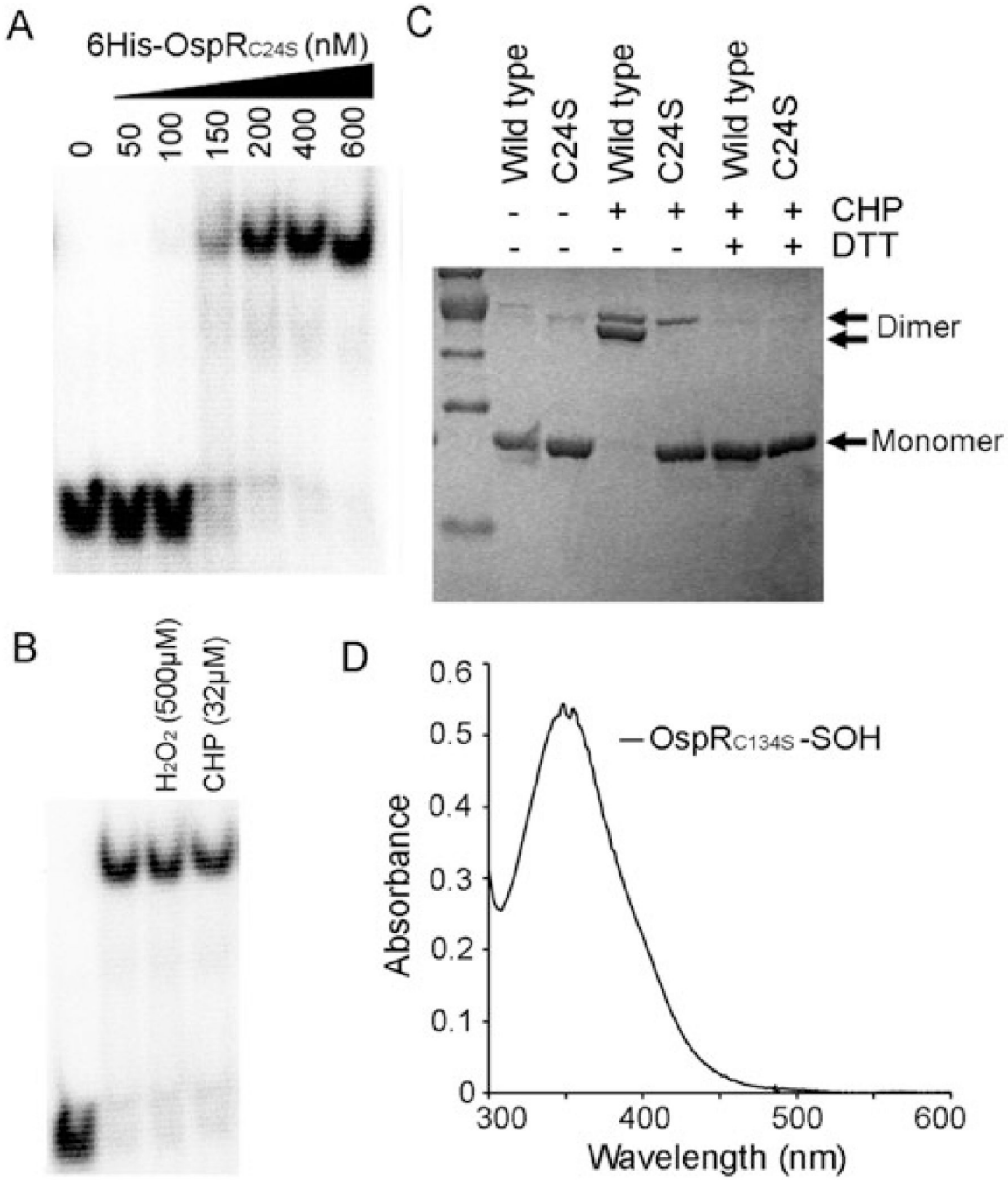

Fig. 5.

Gel shift assay and oxidation of Cys-24 *in vitro*.

A. A gel shift experiment showing binding of 6His-$OspR_{C24S}$ to the *PA2825* promoter DNA.

B. Addition of H_2O_2 or CHP failed to dissociate 6His-$OspR_{C24S}$ from the *PA2825* promoter DNA; 300 nM 6His-$OspR_{C24S}$ was used in each reaction except for lane 1.

C. Disulphide bond formation in OspR as monitored by non-reducing SDS-PAGE. Protein samples were treated with or without CHP for 10 min prior to analysis, as described in *Experimental procedures*. When indicated, 50 mM DTT was added before samples were run on SDS-PAGE. The first lane in the panel contains molecular mass standards corresponding to 15, 25, 32, 50 and 75 kDa (bottom to top).

D. Cys-24-sulphenic acid formation *in vitro* through oxidation with four equivalents (per $OspR_{C134S}$ monomer) of CHP as indicated by the NBD-Cl assay. Cys-S(O)-NBD absorbs at 347 nm.

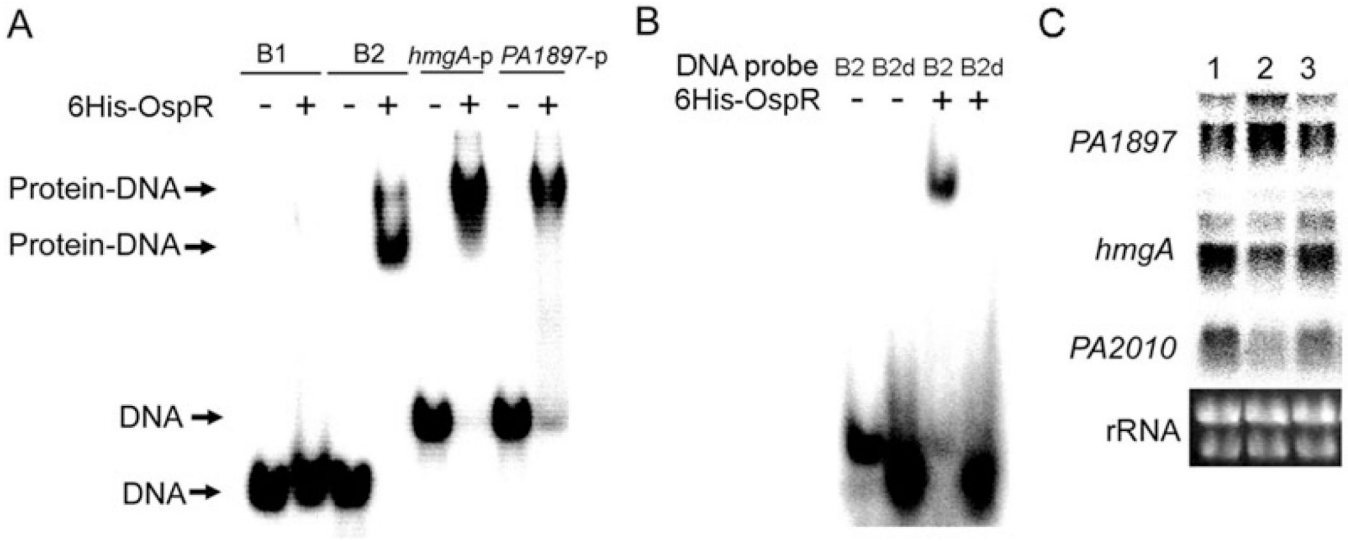

Fig. 6.

Electrophoretic mobility shift assay and Northern blot analysis.

A. Purified wild-type OspR could bind radiolabelled nucleotide containing *PA2009–PA2010* intergenic region and *PA1897* promoter region respectively (see *Experimental procedures*).

B. Electrophoretic mobility shift assay showing that the putative OspR binding site (AATTnAATT) is required for the binding of OspR to the *PA2826* promoter sequence (B2).

C. Northern blot analysis for *hmgA PA2010* and *PA1897*. The overnight culture was diluted 100-fold in fresh LB medium. Bacteria were harvested after incubating at 37°C for 9 h (OD_{600} = 2.5) and then subjected to total RNA isolation. 1: MPAO1/pAK1900; 2: Δ*ospR*/pAK1900; 3: Δ*ospR*/p-*ospR*.

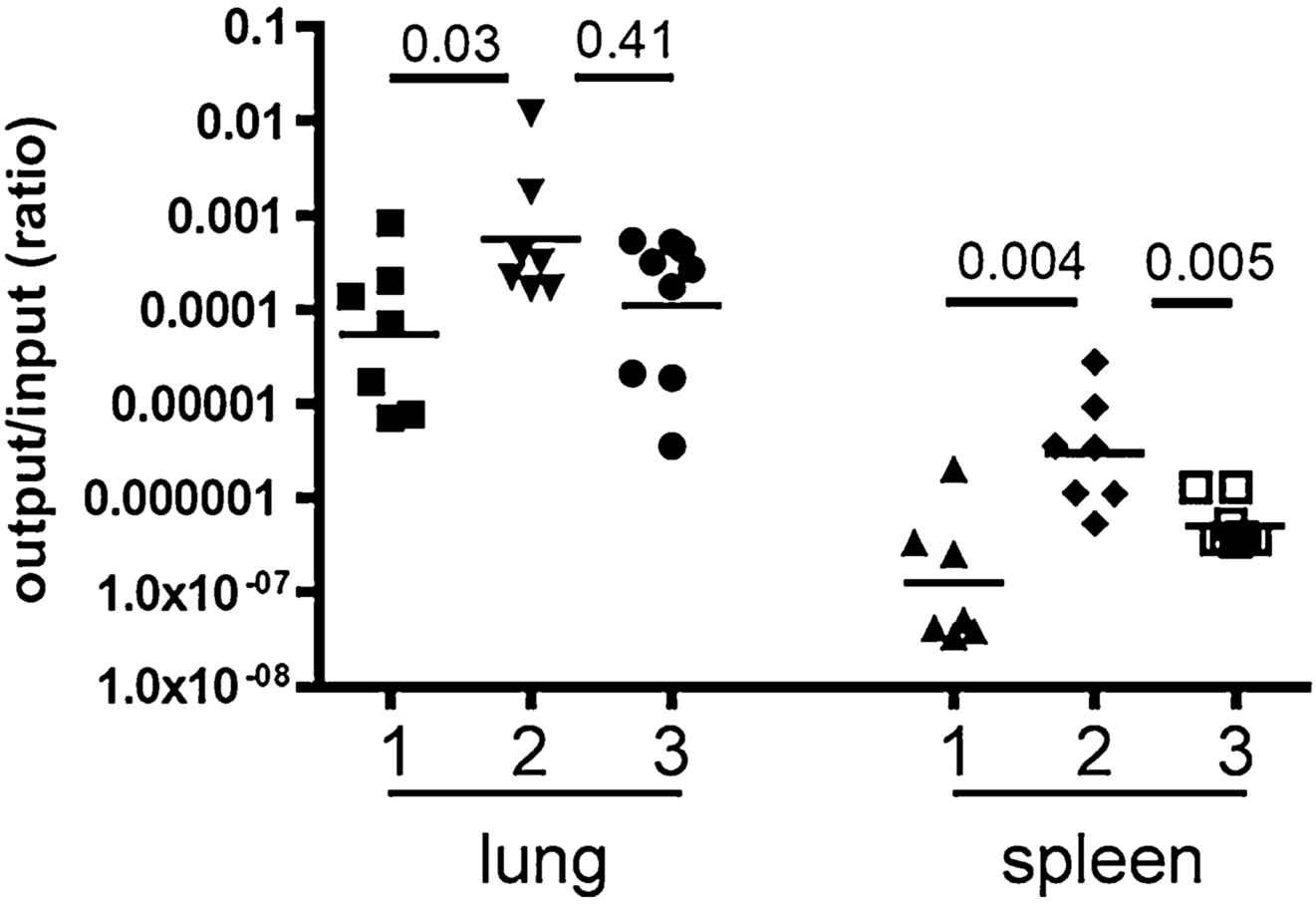

Fig. 7.

Recovery of *P. aeruginosa* derivatives in a mouse model of acute pneumonia. Lightly anaesthetized 8-week-old female C57BL/6 mice were infected with approximately 2×10^7 cfu (colony-forming units) of each bacterial strain. The mice were euthanized 18 h post infection, and the lungs and spleens were each removed, homogenized and re-suspended in PBS plus 0.1% Triton X. Serial dilutions of organ suspensions were plated on Vogel-Bonner minimal medium to determine cfu per organ. Results are expressed as the ratio of cfu recovered per organ (output) to cfu present in the initial inoculum (input) and represent results from $n = 7$–10 mice per strain; the line shows the geometric mean for each group. The Mann–Whitney test was used to calculate *P*-values (two-tailed). 1: MPAO1/pAK1900; 2: Δ*ospR*/pAK1900; 3: Δ*ospR*/p-*ospR*.

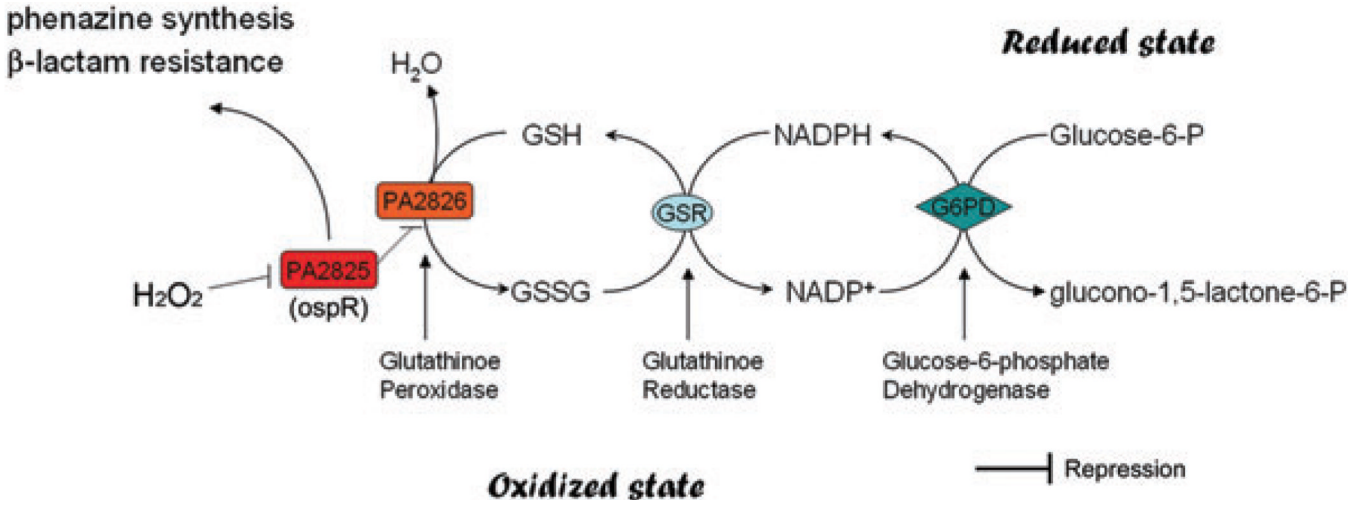

Fig. 8.

Proposed function of *ospR* in *P. aeruginosa* MPAO1. In *P. aeruginosa*, OspR senses oxidative stress and regulates the intracellular redox state through de-repression of *PA2826*. OspR also affects phenazine biosynthesis and β-lactam resistance in *P. aeruginosa* through pathways independent of *PA2826*. GSH, glutathione; GSSG, glutathione disulphide; NADP, nicotinamide adenine dinucleotide phosphate.

Table 1

Promoters identified by consensus sequence search.

Genes	Start[a]	End[a]	Sequence[b]
NP_250033.1|PA1342	−202	−194	tccaAATTtAATTtctt
NP_250034.1|PA1343	−258	−250	aagaAATTaAATTtgga
NP_250564.1|PA1873	−378	−370	gaaaAATTcAATTtaga
NP_250565.1|PA1874	−131	−123	tctaAATTgAATTtttc
NP_250588.1|PA1897	−247	−239	tgacAATTtAATTcgac
NP_250699.1|hmgA/PA2009	−88	−80	gcgtAATTgAATTacga
NP_250700.1|PA2010	−81	−73	tcgtAATTcAATTacgc
NP_251516.1|PA2826	−56	−48	gtgcAATTgAATTgcga
NP_252252.1|PA3562	−261	−253	tgccAATTtAATTaagt
NP_252253.1|fruR/PA3563	−75	−67	acttAATTaAATTggca
NP_254148.1|PA5461	−226	−218	gaaaAATTgAATTcctg

[a] Upstream regions from the start codon.

[b] Uppercase letters indicate the conserved inverted repeat.

Table 2

Bacterial strains and plasmids used in this study.

Plasmids or strains	Genetype, relevant characteristics	Source
Plasmids		
pET28a	T7 *lac* promoter–operator, N-terminal His tag, kan^r	Novagen
pAK1900	*E. coli–P. aeruginosa* shuttle cloning vector, Ap^r Cb^r	Jansons *et al*. (1994)
pJQ200mp18	Gene replacement vector, mob$^+$*sacB*, Gm^r	Quandt and Hynes (1993)
pEX18Ap	Gene replacement vector, mob$^+$*sacB*, Ap^r	Hoang *et al*. (1998)
pPS858	pBR322 derivative carrying a FRT-Gm cassette, Ap^r	Hoang *et al*. (1998)
p-*ospR*	PAk 1900 derivative carrying *ospR*(*PA2825*) on a *c*. 0.9 kb HindIII/BamHI fragment in same orientation as p*lac*	This study
p-*ospR*$_{C24S}$	p-*ospR* derivative carrying serine substitution mutant at the site C24	This study
p-*ospR*$_{C134S}$	p-*ospR* derivative carrying serine substitution mutant at the site C134	This study
p-*ospRC24SC134S*	p-*ospR* derivative carrying serine substitution mutant at the site C24 and C134	This study
p-*PA2826*	PAk 1900 derivative carrying *PA2826* on a *c*. 0.7 kb HindIII/BamHI fragment in same orientation as p*lac*	This study
pET28a–His-ospR	pET28a derivative carrying *ospR*(*PA2825*)	This study
pET28a–His-ospR$_{C24S}$	pET28a derivative carrying *ospR*(*PA2825*) which has serine substitution mutant at the site C24	This study
pET28a–His-ospR$_{C134S}$	pET28a derivative carrying *ospR*(*PA2825*) which has serine substitution mutant at the site C134	This sduty
pJQ200mp18::2825UTD	pJQ200mp18 derivative, for replacing MPAO1 *ospR*(*PA2825*) gene with a tetracycline resistance cassette	This study
pEX18Ap::26-25UGD	pEX18Ap derivative, for replacing MPAO1 *PA2826–2825* locus with a gentamicin resistance cassette	This study
Strains		
Pseudomonas aeruginosa		
MPAO1	Wild type	Jacobs *et al*. (2003)
Δ*ospR*	*PA-MgrA*::Tet^r; MPAO1 derivative with a tetracycline resistance cassette replaced the *ospR*(*PA2825*) gene	This study
Δ*PA2826-ospR*	*PA2826-ospR*::Gen^r, MPAO1 derivative with a gentamicin resistance cassette replaced the *PA2826-ospR* locus	This study
MPAO1/PAK1900	MPAO1 carrying plasmid PAK1900	This study
MPAO1/p-*ospR*	MPAO1 carrying plasmid p-*ospR*	This study
MPAO1/p-*PA2826*	MPAO1 carrying plasmid p-*PA2826*	This study
Δ*ospR*/PAK1900	Δ*ospR* carrying PAK1900	This study
Δ*ospR*/p-*ospR*	Δ*ospR* carrying p-*ospR*	This study
Δ*PA2826-ospR*/PAK1900	Δ*PA2826-ospR* carrying PAK1900	This study
Δ*PA2826-ospR*/p-*ospR*	Δ*PA2826-ospR* carrying p-*ospR*	This study
Δ*PA2826-ospR*/p-*PA2826*	Δ*PA2826-ospR* carrying p-*PA2826*	This study
E. coli		
DH5a	*endA hsdR17 supE44 thi-1 recA1 gyrA relA1* Δ(*lacZYA-argF*)*U169 deoR* (φ*80dlac*Δ(*lacZ*)*M15*)	Lab stock
BL21	F$^-$*ompT hsdS*$_B$ (r_B m_B) *gal dcm met* (DE3)	Lab stock