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Pseudomonas aeruginosa MdaB and WrbA are Water-soluble Two-electron Quinone Oxidoreductases with the Potential to Defend against Oxidative Stress

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Water-soluble quinone oxidoreductases capable of reducing quinone substrates via a concerted two-electron mechanism have been implicated in bacterial antioxidant defence. Two-electron transfer avoids formation of dangerously reactive semi-quinone intermediates, moreover previous work in *Pseudomonas putida* indicated a direct protective effect for the quinols generated by an over-expressed oxidoreductase. Here, the *Pseudomonas aeruginosa* orthologs of five quinone oxidoreductases – MdaB, ChrR, WrbA, NfsB, and NQO1 – were tested for their possible role in defending *P. aeruginosa* against H_2O_2 challenge. In *in vitro* assays, each enzyme was shown to reduce quinone substrates with only minimal semi-quinone formation. However, when each was individually over-expressed in *P. aeruginosa* no overt H_2O_2-protective phenotype was observed. It was shown that this was due to a masking effect of the *P. aeruginosa* catalase, KatA; in a *katA* mutant, H_2O_2 challenged strains over-expressing the WrbA and MdaB orthologs grew significantly better than the empty plasmid control. A growth advantage was also observed for H_2O_2 challenged *P. putida* strains over-expressing *P. aeruginosa wrbA*, *mdaB* or *katA*. Despite not conferring a growth advantage to wild type *P. aeruginosa*, it is possible that these quinone oxidoreductases defend against H_2O_2 toxicity at lower concentrations.

Keywords: NAD(P)H dependent oxidoreductase, quinone reductase, hydrogen peroxide, KatA, *Pseudomonas putida*

Introduction

In biological systems, quinones can act as a double-edged sword. On the one hand they have the propensity to generate high levels of oxidative stress, exacerbated by the activities of one-electron reductases such as lipoyl dehydrogenase (Gonzalez *et al*., 2005) and xanthine oxidase (Nakamura *et al*., 1978), which generate semi-quinone intermediates during reduction. In the presence of molecular oxygen these semi-quinones rapidly cycle back to their oxidized quinone form, generating superoxide radicals in the process. Superoxide can be converted or disproportionate to hydrogen peroxide which, in the presence of iron or other Fenton catalysts, can in turn yield highly damaging hydroxyl radicals (Cadenas *et al*., 1992; Valko *et al*., 2005). The cytotoxicity of these various reactive oxygen species can be so potent that some organisms synthesize and secrete high levels of partially water soluble quinones to poison competitors or predators (e.g., production of the menadione analogues plumbagin and juglone by various plants; Søballe and Poole, 1999).

On the other hand, two-electron reduced quinones (quinols) that are redox stable (i.e., do not autoxidize to generate superoxide) are able to scavenge and detoxify reactive oxygen species (Søballe and Poole, 1999; Ross and Siegel, 2004). Thus, reductases that employ a simultaneous two electron transfer mechanism for quinone reduction not only avoid formation of highly reactive semi-quinones, but may also play key roles in maintaining a pool of reduced quinols that contribute to antioxidant defence (Cadenas *et al*., 1992; Søballe and Poole, 2000).

Mammalian NQO1 (also known as DT-diaphorase) is a water-soluble enzyme that has been known for many years to play this dual antioxidant role (Lind *et al*., 1982; Landi *et al*., 1997). However, the first demonstration that bacteria also possess water-soluble two-electron quinone oxidoreductases capable of making an overt contribution to oxidative stress resistance did not come until much later. Wang and Maier (2004) showed that a *Helicobacter pylori* mutant lacking the NADPH-dependent quinone oxidoreductase MdaB was not only greatly sensitized to oxygen, hydrogen peroxide and the superoxide-generating agent paraquat, but also impaired in its ability to colonize mouse stomachs. Subsequently, over-expression of the unrelated quinone oxidoreductase ChrR [an NAD(P)H-dependent flavoenzyme first characterised as a chromate reductase; Ackerley *et al*. (2004)] was found to substantially increase the tolerance of *Pseudomonas putida* to H_2O_2 challenge (Gonzalez *et al*., 2005). Several other water-soluble NAD(P)H-dependent bacterial enzymes have also been characterised as divalent quinone oxidoreductases and proposed to have antioxidant potential, including *Escherichia coli* WrbA, NfsA, and NfsB, however prior to this work no conclusive role in oxidative stress resistance had been demonstrated for any of these (Zenno *et al*., 1996;

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Liochev *et al.*, 1999; Patridge and Ferry, 2006; Andrade *et al.*, 2007; Carey *et al.*, 2007). The importance of biochemical data in bridging the gap between putative and definitive assignation of function for enzymes such as quinone oxidoreductases has been commented on, with specific reference to WrbA (White, 2006).

During infection the opportunistic human pathogen *Pseudomonas aeruginosa* is exposed to considerable oxidative stress, in particular from superoxide and hydrogen peroxide generated by phagocytes (Rossi *et al.*, 1979; Sadikot *et al.*, 2005). As H_2O_2 is uncharged it is more able than $O_2^{\cdot-}$ to cross membranes, making it more likely to be the primary antibacterial agent (Rossi *et al.*, 1979). That the ability to guard against H_2O_2 challenge is an important consideration during pathogenesis was highlighted by identification of the *P. aeruginosa* major catalase, KatA, as a virulence factor (Lee *et al.*, 2005). However, KatA has a very high K_m for H_2O_2 (45 mM) and at low H_2O_2 concentrations the reaction rate drops by several orders of magnitude (Lee *et al.*, 2005). Thus, we hypothesized that alternative antioxidant systems with a greater affinity for H_2O_2 might also contribute to virulence. The other *P. aeruginosa* catalases, KatB and KatE, do not appear to fulfil this role (Ma *et al.*, 1999; Lee *et al.*, 2005). We therefore decided to test whether soluble two-electron quinone oxidoreductase enzymes might also contribute to H_2O_2 resistance in *P. aeruginosa*.

Materials and Methods

Bacterial strains, plasmids, and other materials

All chemicals, reagents and media used in this study were obtained from Sigma-Aldrich (USA) or Thermo Fisher Scientific (USA) unless otherwise stated. Restriction enzymes were supplied by New England Biolabs (NEB; USA), T4 DNA ligase was by Invitrogen (USA), and Phusion™ high-fidelity DNA polymerase by Finnzymes (Finland). *P. aeruginosa* PAO1 was generously supplied by Prof. Iain Lamont (University of Otago) and *P. putida* KT2440 and the isogenic *chrR* mutant by Prof. AC Matin (Stanford University). The IPTG-inducible broad host range expression plasmid pSX (Owen and Ackerley, 2011) was used for gene over-expression in *P. aeruginosa* and *P. putida* (yielding clearly visible bands for each over-expressed protein on Coomassie Brilliant Blue stained SDS-PAGE gels); and the His_6-tagged expression vector pET28a$^+$ (Novagen, Merck, Germany) was used for protein purification.

Identification and cloning of *katA* and quinone oxidoreductase genes

Protein homology searches were carried out using the alignment search algorithm BLASTP (default parameters), hosted by the *Pseudomonas* Genome Database (Winsor *et al.*, 2011), with the sequences of MdaB from *H. pylori* ATCC 43504 (Wang and Maier, 2004); ChrR from *P. putida* KT2440 (Gonzalez *et al.*, 2005); NQO1 from *Homo sapiens* (Jaiswal, 1991); and WrbA, NfsA and NfsB from *E. coli* W3110 (Prosser *et al.*, 2010). With the exception of NfsA (no hit sharing >20% identity across the full amino acid sequence), the best aligned predicted protein sequence from the *P. aeruginosa* genome was taken forward for further study (**PA0949**, 39% amino acid identity with WrbA; **PA1204**, 46% identity with ChrR; **PA2580**, 55% identity with MdaB; **PA4975**, 35% identity with NQO1; and **PA5190**, 25% identity with NfsB; Green *et al.*, 2013). Primers for amplifying and cloning *nfsB_Pa, nqo1_Pa* and *chrR_Pa* into pSX and pET28a$^+$ were as described in Prosser *et al.* (2013), and for *mdaB_Pa, wrbA_Pa*, and *P. aeruginosa katA* (**PA4236**) the following primer sets were used: mdaB_Fw (CCC**CATATG**AAAAACATTC TCCTGC), mdaB_Rv (CCC**GTCGAC**TCAGCCG GCGC); wrbA_Fw (CCC**CATATG**TTGAGCAGTCCCTACATCCT), wrbA_Rv (CCC**GTCGAC**TCAACTCCCCAGCTTGCCGG); and katA_Fw (CCC**CATATG**GAAGAGAAGACCCGCCT), katA_Rv (CCC**GCGGCCGC**TCAGTCCAGCTTCAGGCC GA) (restriction sites used for cloning are in bold). For *P. aeruginosa katA* gene knockout a two-step overlap PCR was used to generate an in-frame markerless truncated *katA* gene construct consisting of fused 500 bp sequences flanking either side of the wild type *katA* gene, using the following primer pairs: katAKO_UL (CCC**TCTAGA**GACAGCGTCGC CAACCGTCG), katAKO_LL (CTCTTCCATTTACTCTCT CCTCAAC); and katAKO_UR (GTTGAGGAGAGAGTA AATGGAAGAGCTGGACTGATGGCCTGATGA), katAKO_LR (CCC**TCTAGA**CCAACTGGGCCTTGAGGA TG) (restriction sites used for cloning the overlap product are in bold, and the overlap homology region is underlined). The truncated gene construct was used for *katA* gene knockout via in a two-step suicide plasmid protocol as previously described (Ackerley and Lamont, 2004).

Protein purification

Recombinant His_6-tagged quinone oxidoreductases were expressed from pET28a$^+$ in *E. coli* BL21(DE3) and purified by nickel-affinity chromatography using the His·Bind Kit (Novagen, Merck). Eluted proteins were incubated with 100 mM FMN cofactor (FAD cofactor for MdaB_Pa) at 4°C overnight, to restore cofactor lost during the purification process, followed by desalting into 40 mM Tris-Cl, pH 7.0 [as described in Prosser *et al.* (2010)]. Quantification of purified protein was performed using the Dc™ protein assay kit (Bio-Rad, USA) against BSA standards.

Enzyme assays

Quinone oxidoreductase activity was measured spectrophotometrically as described (Gonzalez *et al.*, 2005), following diminishing NADH (NADPH for MdaB_Pa) absorbance at 340 nm at room temperature. One milliliter reaction mixtures consisted of 25–250 μM quinone substrate and 0.1–5.0 μg of purified recombinant enzyme in 50 mM Tris-Cl buffer pH 7.4, and reactions were initiated by the addition of 250 mM NAD(P)H. Benzoquinone was added from 10 mM stock in double-distilled water, menadione from 10 mM stock dissolved in ethanol, and coenzyme Q1 from a 200 mM stock in N,N-dimethylformamide that was subsequently diluted to a 10 mM working stock in ethanol. For steady-state kinetic analysis, non-linear regression and Michaelis-Menten curve fitting was performed using GraphPad™ Prism 5 (GraphPad Software, USA). To detect one-electron transfer,

Table 1. Steady statE Michaelis-Menten kinetic parameters with three quinone substrates

Protein	Substrate	V_{max} (μmol/min/mg)[a,b]	K_m (μM)[a,b]	k_{cat} (s^{-1})[a,b]	k_{cat}/K_m ($M^{-1}s^{-1}$)[b]
NQO1_Pa	Benzoquinone	610 ± 80	320 ± 50	410 ± 50	$1.3x10^6$
	Menadione	0.03 ± 0.01	1400 ± 480	0.02 ± 0.01	$1.6×10^1$
	Coenzyme Q1	N.D.[c]	N.D.[c]	N.D.[c]	N.D.[c]
ChrR_Pa	Benzoquinone	870 ± 60	290 ± 40	590 ± 40	$2.0×10^6$
	Menadione	190 ± 20	780 ± 90	130 ± 10	$1.6×10^5$
	Coenzyme Q1	15 ± 5	1500 ± 600	10 ± 3	$6.8×10^3$
MdaB_Pa	Benzoquinone	0.1 ± 0.04	0.3 ± 0.28	0.1 ± 0.03	$3.0×10^5$
	Menadione	0.1 ± 0.02	0.4 ± 0.27	0.1 ± 0.01	$2.2×10^5$
	Coenzyme Q1	0.4 ± 0.29	2.6 ± 2.3	0.2 ± 0.2	$1.4×10^5$
NfsB_Pa	Benzoquinone	0.03 ± 0.01	0.9 ± 0.4	0.02 ± 0.01	$2.3×10^4$
	Menadione	0.03 ± 0.01	2.6 ± 0.8	0.02± 0.01	$6.8×10^3$
	Coenzyme Q1	N.D.[c]	N.D.[c]	N.D.[c]	N.D.[c]
WrbA_Pa	Benzoquinone	0.1 ± 0.03	1.5 ± 0.8	0.04 ± 0.02	$3.4×10^4$
	Menadione	N.D.[c]	N.D.[c]	N.D.[c]	N.D.[c]
	Coenzyme Q1	N.D.[c]	N.D.[c]	N.D.[c]	N.D.[c]

[a] These are apparent values as measured at 250 μM NADH
[b] All values for V_{max}, K_m, and k_{cat} represent the mean of three replicates ± 1 standard deviation
[c] No activity detected

reaction mixtures consisted of 0.1–5.0 μg of purified quinone oxidoreductase proteins or control lipoyl dehydrogenase from *Clostridium kluyveri* (Sigma-Aldrich, USA), 50 μM benzoquinone, and 75 μM cytochrome *c* in 50 mM K phosphate buffer pH 6.0 [the low pH required to avoid quinone autoxidation; Gonzalez *et al.* (2005)]. Reactions were activated by addition of 250 μM NAD(P)H and monitored spectrophotometrically at 550 nm for 3.5 min, following the appearance of reduced cytochrome *c* (this time period was found to be sufficient for complete benzoquinone reduction in separate control reactions, as monitored spectrophometrically at 340 nm, following NAD(P)H consumption).

Results and Discussion

To determine whether soluble two-electron quinone oxidoreductases might also make a discernible contribution to defending *P. aeruginosa* against H_2O_2 challenge we first identified plausible enzyme candidates encoded within the *P. aeruginosa* PAO1 genome (Stover *et al.*, 2000) by BLAST searching against the amino acid sequences of human NQO1, *P. putida* ChrR, *H. pylori* MdaB, and *E. coli* WrbA, NfsA, and NfsB. No clear orthologs of *E. coli* NfsA were identified, however orthologs sharing at least 25% amino acid identity in a full sequence alignment with each of the other enzymes were identified and selected for further analysis (details in 'Materials and Methods'). For convenience, each of these enzymes is referred to by their orthologous name followed by the suffix _Pa, e.g. MdaB_Pa for *P. aeruginosa* gene product 2580, which shares 55% amino acid identity with *H. pylori* MdaB.

Genes encoding each of the five quinone oxidoreductase candidates were amplified by PCR and cloned in-frame into pET28a+, enabling their individual expression and purification as recombinant His_6-tagged proteins by Ni-NTA chromatography. As previously described by Gonzalez *et al.* (2005) for *P. putida* ChrR, Michaelis-Menten steady state kinetics were then measured for each purified enzyme with each of three quinone substrates, exhibiting varying degrees of water solubility: benzoquinone (most soluble, logP = 0.27), menadione (intermediate solubility, logP = 1.6), and coenzyme Q1 (least soluble, logP = 2.2) (Table 1). Whereas all five enzymes were active with benzoquinone, they varied widely in their ability to metabolise the more lipophilic quinone substrates. The most active enzymes with benzoquinone, NQO1_Pa and ChrR_Pa, had specificity constants that were comparable to previously reported values for the H_2O_2-quenching ChrR of *P. putida* [(k_{cat}/K_M = 8.5 × 10^6 $M^{-1}sec^{-1}$; Gonzalez *et al.* (2005)]. The remaining enzymes were also able to reduce benzoquinone but at substantially reduced rates compared to NQO1_Pa and ChrR_Pa. However, MdaB_Pa was able to reduce the least soluble substrate, coenzyme Q1, with a k_{cat}/K_M ~20-fold higher than ChrR_Pa [and ~3-fold higher than *P.*

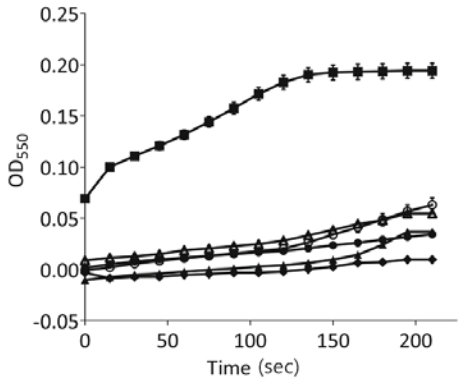

Fig. 1. Reduction of cytochrome *c* monitored spectrophotometrically at 550 nm during the reduction of 50 μM benzoquinone catalysed by NQO1_Pa (l), ChrR_Pa (●), MdaB_Pa (○), NfsB_Pa (▲), WrbA_Pa (◆), or *C. kluyveri* lipoyl dehydrogenase (■). The appearance of reduced cytochrome c during the lipoyl dehydrogenase reaction indicates at least partial one-electron transfer, whereas the minimal appearance of this species in the *P. aeruginosa* quinone oxidoreductase reactions signifies a predominantly two-electron mode of quinone reduction.

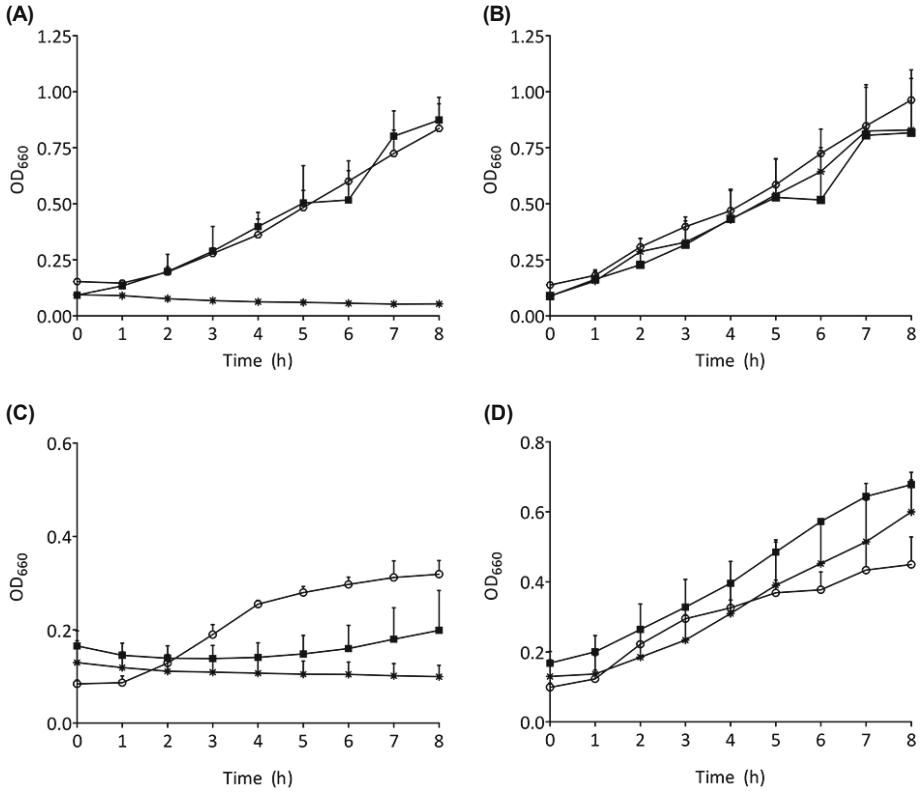

Fig. 2. A powerful KatA catalase renders *P. aeruginosa* PAO1 substantially more resistant to H_2O_2 challenge than *P. putida* KT2440. (A, B) Eight hour growth curves (OD_{660}) for wild type *P. aeruginosa* PAO1 + pSX (■), the *P. aeruginosa ΔkatA* mutant + pSX (∗), and *P. aeruginosa ΔkatA* mutant complemented with a *katA* gene copy over-expressed from pSX (○) in the presence of either 100 mM H_2O_2 (A) or unamended media (B). (C, D) Eight hour growth curves (OD_{660}) for wild type *P. putida* KT2440 + pSX (■), the *P. putida ΔchrR* mutant + pSX (∗), and *P. putida ΔchrR* mutant complemented with a *katA* gene copy over-expressed from pSX (○) in the presence of 6 mM H_2O_2 (C) or unamended media (D). All strains were inoculated to an initial OD_{660} of 0.1 in LB media amended with 0.25 mM IPTG and 10 μg/ml gentamycin, ± H_2O_2 as indicated, and the culture OD_{660} was measured every hour for 8 h during growth at 37°C with shaking at 250 rpm. The plotted data are the means of 3 independent experiments, each performed in duplicate, and error bars are ± 1 standard deviation.

putida ChrR; Gonzalez *et al.* (2005)]. Although the identities of the quinone substrate or substrates that mediate H_2O_2-quenching in bacteria are unknown, it has previously been speculated that coenzyme Q1 could play a role in this process (Søballe and Poole, 1999).

With all candidate enzymes having been shown to actively reduce quinone substrates, the mode of electron transfer was determined for each by implementation of a cytochrome *c* trap. In liquid assays cytochrome *c* is rapidly reduced by benzo-semi-quinone, but not by benzoquinol; and this reduction leads to a quantifiable increase in absorbance at 550 nm that can be monitored spectrophotometrically (Gonzalez *et al.*, 2005). During complete reduction of benzoquinone by each of the *P. aeruginosa* quinone oxidoreductases, only trace levels of reduced cytochrome *c* were detected, indicating primarily two-electron reduction mechanisms that avoid formation of benzosemiquinone intermediates (Fig. 1). In contrast, lipoyl dehydrogenase (an obligate one-electron reducer from *Clostridium kluyveri*) generated high levels of reduced cytochrome *c* during benzoquinone reduction (Fig. 1).

The primarily two-electron reduction mechanism exhibited by each *P. aeruginosa* quinone oxidoreductase *in vitro* was consistent with that previously observed for *P. putida* ChrR (Gonzalez *et al.*, 2005), and encouraged us to investigate whether one or more of these enzymes might similarly confer protection against oxidative stress *in vivo*. To test this we chose to individually over-express each candidate gene in *P. aeruginosa* PAO1, reasoning that the possibility of functional redundancy between these different quinone oxidoreductases might confound the analysis of individual gene deletions, and that in the previous *P. putida* study, *chrR* over-expression had yielded a more profound change in the H_2O_2 resistance phenotype than *chrR* gene deletion (Gonzalez *et al.*, 2005). The genes encoding each of the five quinone oxidoreductase candidates were transferred into the broad host range IPTG-inducible expression plasmid pSX (Owen *et al.*, 2011), and the resulting constructs used to transform *P. aeruginosa* PAO1.

Preliminary trials to establish appropriate H_2O_2 concentrations for *P. aeruginosa* growth assays revealed that *P. aeruginosa* PAO1 is substantially more resistant to H_2O_2 challenge than the *P. putida* KT2440 strain subsequently employed in the ChrR study (Gonzalez *et al.*, 2005). Whereas *P. aeruginosa* exhibited substantial tolerance to H_2O_2 at concentrations in excess of 100 mM (Figs. 2A and 2B), *P. putida* challenged with only 6 mM H_2O_2 did not progress beyond its initial OD_{660} of inoculation during an 8 h incubation period (Figs. 2C and 2D). No clear protective effect was evident for any of the over-expressed oxidoreductases at any of the H_2O_2 concentrations tested (ranging from 100 mM to 0.4 mM). This led us to consider whether the *P. aeruginosa* catalase, KatA, might be masking any visible phenotype. When we deleted the *katA* gene from *P. aeruginosa* PAO1 the resulting strain was greatly sensitized to H_2O_2, whereas complementation of this mutation by a *katA* gene copy over-expressed from pSX restored the highly H_2O_2 resistant phenotype (Figs. 2A and 2B). Likewise, over-expression of the *P. aeruginosa katA* catalase gene in the *chrR* mutant of *P. putida* KT2440 increased the resistance of this bacterium to H_2O_2 (Figs. 2C and 2D), albeit not to the same levels as the complemented *P. aeruginosa katA* mutant (Fig. 2A).

We next sought to determine whether over-expression of

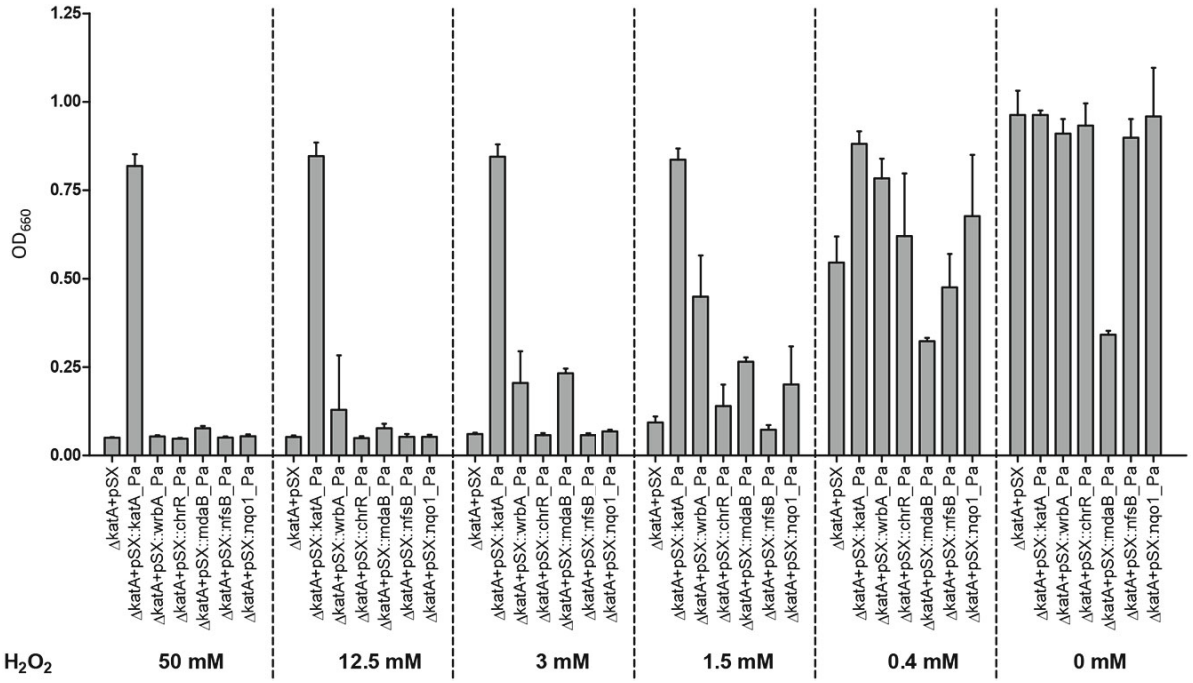

Fig. 3. The effect of quinone oxidoreductase or *katA* over-expression in the *ΔkatA* mutant of *P. aeruginosa* PAO1. Strains of the *P. aeruginosa ΔkatA* mutant over-expressing either *P. aeruginosa katA*, *wrbA_Pa*, *mdaB_Pa*, *chrR_Pa*, *nfsB_Pa*, or *NQO1_Pa* from plasmid pSX, or transformed with a pSX empty plasmid control, were inoculated to an initial OD_{660} of 0.1 in LB media amended with 0.25 mM IPTG, 10 μg/ml gentamycin, and H_2O_2 as indicated. Cultures were incubated at 37°C with shaking at 250 rpm and growth was monitored by hourly OD_{660} readings for 8 h. Bars indicate end-point growth measured by OD_{660} at 8 h post treatment, and are the means of 3 independent experiments, each performed in duplicate, with error bars ± 1 standard deviation.

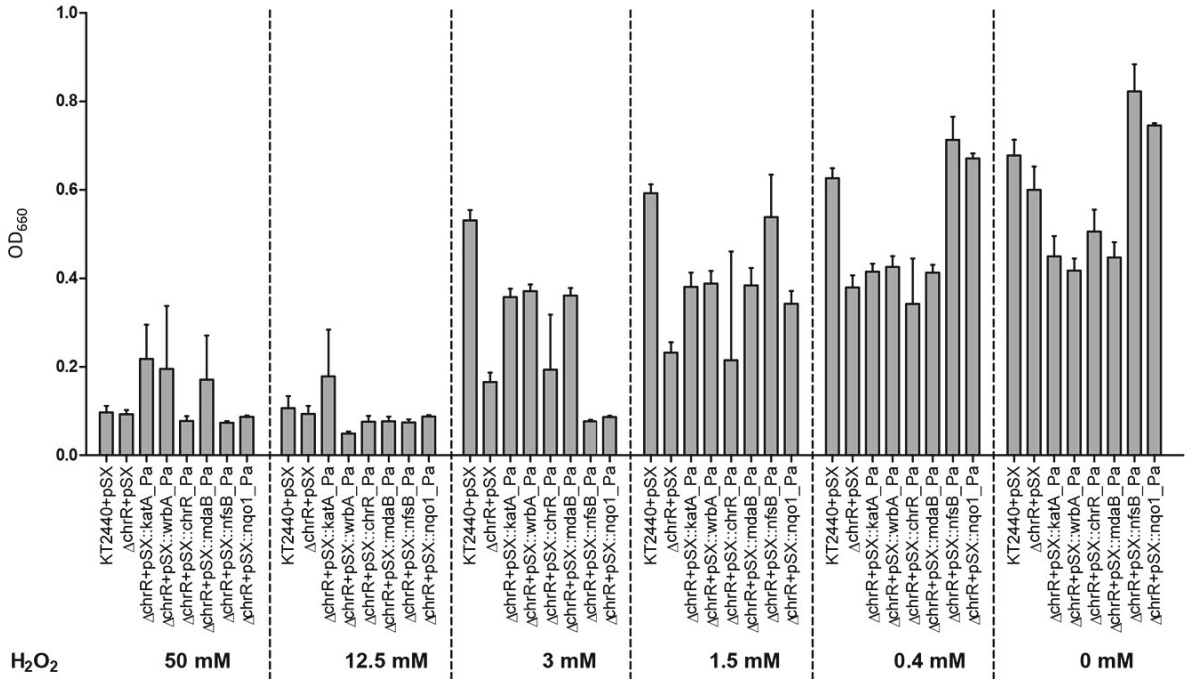

Fig. 4. The effect of quinone oxidoreductase or *P. aeruginosa katA* over-expression in the *ΔchrR* mutant of *P. putida* KT2440. Strains of the *P. putida ΔchrR* mutant over-expressing either *P. aeruginosa katA, wrbA_Pa*, *mdaB_Pa*, *chrR_Pa*, *nfsB_Pa*, or *NQO1_Pa* from plasmid pSX, as well as the wild type and *ΔchrR* mutant transformed with a pSX empty plasmid control, were inoculated to an initial OD_{660} of 0.1 in LB media amended with 0.25 mM IPTG, 10 μg/ml gentamycin, and H_2O_2 as indicated. Cultures were incubated at 37°C with shaking at 250 rpm and growth was monitored by hourly OD_{660} readings for 8 h. Bars indicate end-point growth measured by OD_{660} at 8 h post treatment, and are the means of 3 independent experiments, each performed in duplicate, with error bars ± 1 standard deviation.

each *P. aeruginosa* quinone oxidoreductase in the *katA* mutant could provide some measure of compensation for the loss of catalase activity. Each over-expression plasmid was transformed into the *katA* mutant, and replicate cultures of the resulting strains were grown for eight hours across a range of H_2O_2 challenge concentrations. Although none of the quinone oxidoreductases were able to afford similar levels of protection to KatA, the strain over-expressing *wrbA_Pa* grew significantly better than the empty plasmid *katA* mutant control at both 3 mM and 1.5 mM of H_2O_2 challenge ($p<0.05$ and $p<0.01$ respectively; one-way ANOVA) (Fig. 3). Likewise, the strain over-expressing *mdaB_Pa* was significantly more resistant than the *katA* mutant control at 3 mM H_2O_2 ($p<0.05$) and also grew better at 1.5 mM H_2O_2 (Fig. 3). As over-expression of *mdaB_Pa* was found to be detrimental to growth of the unchallenged strain (Fig. 3), it is likely that a direct comparison of growth level with the *katA* control underrepresents significance for this strain.

As an alternative means of testing the antioxidant potential of the *P. aeruginosa* quinone oxidoreductases, we also sought to determine whether any of them were able to complement the previous *chrR* gene deletion mutation in *P. putida* KT2440 (Gonzalez *et al.*, 2005). Although not quite reaching significance at any H_2O_2 concentration (e.g., at 3 mM $p = 0.077$, 0.094 and 0.071, respectively), the strains over-expressing *P. aeruginosa katA, wrbA_Pa* and *mdaB_Pa* consistently grew better than the empty plasmid control under H_2O_2 challenge, but not in unamended growth media (Fig. 4). In contrast, over-expression of the *P. aeruginosa chrR* gene was not able to complement the *P. putida chrR* mutation. This latter observation was consistent with the apparent inability of ChrR_Pa to defend against H_2O_2 challenge in *P. aeruginosa* (Fig. 3), and is possibly indicative of a different biological role to its *P. putida* ortholog. The failure of *P. aeruginosa* KatA to provide a significant level of protection in the *P. putida chrR* mutant was more surprising to us, but may reflect impaired folding and/or partial inhibition of activity in the heterologous cellular environment.

Based on these data we conclude that *P. aeruginosa* contains multiple water-soluble two-electron quinone oxidoreductase enzymes with the potential to make minor contributions to oxidative stress resistance. In particular, this study provides the first demonstration of an antioxidant phenotype for WrbA, a family of enzyme that was previously hypothesized to have this potential but for which a knockout mutant showed no clear phenotype in *E. coli* in Biolog phenotype arrays (Patridge and Ferry, 2006). It is important not to overstate the significance of this finding, as in wild type *P. aeruginosa* any H_2O_2 protective growth phenotype arising from *wrbA* or *mdaB* over-expression was masked by the dominant effect of the powerful KatA catalase. Moreover, in three previous microarray studies where *P. aeruginosa* PAO1 was challenged with between 1 mM and 10 mM H_2O_2 *in vitro*, only expression of the *katA* gene (and not any of the quinone oxidoreductase candidates examined here) was consistently up-regulated (Palma *et al.*, 2004; Chang *et al.*, 2005; Salunkhe *et al.*, 2005). Nonetheless, it is possible that *P. aeruginosa* WrbA and/or MdaB make a physiologically relevant contribution at low concentrations of H_2O_2, where KatA is far less efficient (Lee *et al.*, 2005). At such concentrations, growth may not be substantially impaired, but the cell may still be subject to serious damage. For example, it has been shown that H_2O_2 is more damaging to DNA at low concentrations than high concentrations (Imlay *et al.*, 1988; Nakamura *et al.*, 2003), and *H. pylori* MdaB, which is up-regulated in response to oxidative stress, has been suggested to play a role in mitigating DNA damage (Olczak *et al.*, 2005). Alternatively it is possible that, as previously proposed by Patridge and Ferry (2006) for *E. coli* WrbA, the *P. aeruginosa* quinone oxidoreductases may play a primary role in cell signalling by regulating the redox status of the cellular quinone pool, which can exert far-reaching effects (e.g., Oh and Kaplan, 2000). In this scenario, direct quenching of H_2O_2 by reduced quinones could be a beneficial secondary activity, as well as a mechanism for signal transduction.

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