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ChrR, a Soluble Quinone Reductase of *Pseudomonas putida* That Defends against H_2O_2*

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Most bacteria contain soluble quinone-reducing flavoenzymes. However, no biological benefit for this activity has previously been demonstrated. ChrR of *Pseudomonas putida* is one such enzyme that has also been characterized as a chromate reductase; yet we propose that it is the quinone-reducing activity of ChrR that has the greatest biological significance. ChrR reduces quinones by simultaneous two-electron transfer, avoiding formation of highly reactive semiquinone intermediates and producing quinols that promote tolerance of H_2O_2. Expression of *chrR* was induced by H_2O_2, and levels of *chrR* expression in overexpressing, wild type, and knock-out mutant strains correlated with the H_2O_2 tolerance and scavenging ability of each strain. The *chrR* expression level also correlated with intracellular H_2O_2 levels as measured by protein carbonylation assays and fluorescence-activated cell scanning analysis with the H_2O_2-responsive dye H_2DCFDA. Thus, enhancing the activity of ChrR in a chromate-remediating bacterial strain may not only increase the rate of chromate transformation, it may also augment the capacity of these cells to withstand the unavoidable production of H_2O_2 that accompanies chromate reduction.

Microbial bioreduction of Cr(VI) to Cr(III) is a promising strategy for detoxification of chromate, a prevalent anthropogenic pollutant. A wide range of bacteria, including *Escherichia coli*, *Shewanella oneidensis, Deinococcus radiodurans,* pseudomonads, and several sulfate reducers have been identified that are capable of carrying out a complete reduction of Cr(VI) to Cr(III) (1, 2). However, high cell densities are generally required for significant levels of Cr(VI) transformation, and an effective system for *in situ* bioremediation has yet to be developed. To enhance this activity, we have proposed molecular engineering of cells and enzymes to decrease chromate toxicity to bacteria and increase chromate transformation (3–5).

Previously, we described the purification and characterization of ChrR, a soluble chromate-reducing enzyme from *Pseudomonas putida* (3, 6). ChrR is a dimeric flavin mononucleotide-binding flavoprotein that is able to catalyze a full reduction of Cr(VI) to Cr(III) (6). A *chrR* mutant of *P. putida* was impaired both in chromate transformation rate and viability in the presence of chromate (3) and thus ChrR is a promising candidate for bioengineering studies aimed at generating a more effective chromate-reducing strain. Not only does the identification of individual enzymes that make a discernible contribution to chromate reduction pave the way for directed evolution of an enhanced chromate-reducing enzyme, it also offers scope for regulating the expression of that enzyme in a way that maximizes its chromate-reducing potential.

Several researchers have commented that, as introduction of chromate into the environment is a recent anthropogenic event, enzymatic chromate reduction is likely a secondary activity of reductases with different primary roles (6–9). Affinity chromatography during biochemical purification of chromate-reducing enzymes has identified multiple peaks of chromate-reducing activity in cellular extracts (6, 10), consistent with this hypothesis. That chromate reduction might not be the primary biological role of ChrR does not argue against the potential of this enzyme for bioremediation; however, it is of interest to discern what such a role might be, to better gauge possible consequences of manipulating the activity and expression of this enzyme.

ChrR contains the signature sequence LFVTPEYN*XXXXXX*-LKNAID*XX*S at amino acid positions 75–98 (3), identifying it as a member of the NADH_dh2 family of putative flavin-binding quinone reductases. Although the membrane-bound quinone oxidoreductases have been well studied in respiration, little is known about the biological role of soluble bacterial quinone reductases, which can be either flavin-binding or flavin-independent (11). The flavoenzymes tend to have broad substrate specificity, and have been primarily characterized in the context of alternative substrates (*e.g.* nitroreductase NfsA (12), ferric reductase FerB (13), and several chromate reductases (5)). Even outside of bacteria, only one soluble flavoenzyme has been well characterized as a quinone reductase, namely mammalian NQO1[1] (14). NQO1 is believed to act primarily in an antioxidant capacity, reducing membrane quinones, such as vitamin E and coenzyme Q_{10} divalently to quinols, which then act as quenchers of reactive oxygen species and lipid peroxide radicals (15, 16). ChrR shares no sequence homology with NQO1. Nonetheless, in the work described here, we showed a clear benefit of the quinone reductase activity of ChrR to *P. putida* in guarding against H_2O_2 stress.

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[1] The abbreviations used are: NQO1, NAD(P)H quinone oxidoreductase 1; LB, Luria-Bertani medium; FACS, fluorescence-activated cell sorter; H_2DCFDA, dihydrodichlorofluorescein diacetate; LpDH, lipoyl dehydrogenase.

TABLE I
Bacterial strains and plasmids

	Relevant characteristics	Reference or source
P. putida strains		
KT2440	Wild type genome sequenced strain	7
chrR mutant	Isogenic with wild type	3
chrR$^+$	Overexpression strain; wild type containing pMMB(*chrR*)	4
E. coli strain		
BL21 (*chrR*)	F$^-$ *ompT hsdS*$_B$ ($r_B^- m_B^-$) *gal dcm* (DE3) containing pCHP4	Novagen
Plasmids		
pCHP4	pET28a$^+$ expressing the KT2440 *chrR* gene (NdeI/EcoRI); Km^r	3
pMMB67EH	Broad host range *tac* expression vector; Cb^r	29
pMMB(*chrR*)	pMMB67EH with Bam HI/HindIII His-tagged *chrR* insert	4

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—The strains and plasmids used in this study are listed in Table I. In experiments comparing the wild type and *chrR* mutant to the *chrR*$^+$-overexpressing strain, the former two strains contained the empty plasmid pMMB67EH. LB was used for growth of all strains and was amended with 500 $\mu g \cdot ml^{-1}$ carbenicillin and 0.5 mM isopropyl 1-thio-β-D-galactopyranoside for strains containing pMMB67EH or pMMB(*chrR*). High levels of ChrR were detected in cell extracts from the *chrR*$^+$ strain under these conditions, using the GelCode® His_6 protein tag stain (Pierce) (results not shown). Recombinant ChrR protein was purified from BL21(*chrR*) cells as described previously (3).

Assays—Quinone reductase activity was measured spectrophotometrically by monitoring NADH disappearance at 340 nm. Kinetic measurements of enzyme activity were performed at the optimal pH and temperature for individual quinone substrates, which were determined in separate experiments. Optimal pH was determined using 50 mM potassium phosphate (pH 5.8, 6.0, 6.5, 7.0, 7.5, or 8.0) and Tris/HCl (pH 7.5, 8.0, 8.5, or 8.9) buffers. Optimal temperature was determined in a Shimadzu temperature-controlled spectrophotometer. Protein concentrations were determined with the Bio-Rad Dc protein assay kit, using bovine serum albumin as the standard. One-ml reaction mixtures contained 50 mM buffer, 250 μM quinone substrate (50 μM for coenzyme Q_1), and 0.1–5.0 μg of purified recombinant ChrR, and reactions were initiated by the addition of 250 μM NADH. For the steady state analysis of the reaction mechanism of ChrR, 1-ml reaction mixtures contained 50 mM Tris/HCl buffer, pH 8.0, 0.25 μg of ChrR, and 50–250 μM menadione and were initiated by the addition of either 50, 100, or 250 μM NADH. Benzoquinone was added from a 10 mM stock in double distilled H_2O, menadione and duroquinone from a 10 mM stock dissolved in ethanol, and coenzyme Q_1 from a 200 mM stock in *N*,*N*-dimethylformamide that was subsequently diluted to a 250 μM working stock (with continuous vortexing during dilution) in double distilled H_2O.

The reaction mixture used to investigate semiquinone generation during benzoquinone reduction by the pure ChrR protein or LpDH control (lipoyl dehydrogenase, alternative nomenclature diaphorase, from *Clostridium kluyveri*; purchased from Sigma), with cytochrome *c* as a trap for semiquinone, was as follows: 50 mM potassium phosphate buffer, pH 6.0 (to avoid quinone autoxidation, which occurs spontaneously above pH 7.0), 50 μM benzoquinone, 0.1 $\mu g \cdot ml^{-1}$ ChrR or 2.5 $\mu g \cdot ml^{-1}$ LpDH, and 75 μM cytochrome *c* (from horse heart) (Sigma). Reactions were initiated by the addition of 250 μM NADH. Increase in absorbance because of reduced cytochrome *c* was followed at 550 nm at 30 °C.

Culture growth was monitored spectrophotometrically at 660 nm. H_2O_2 was added to the cultures where indicated. Residual H_2O_2 was assayed with the fluorescent dye Amplex Red (Molecular Probes), and levels of activated dye were quantified in a Turner® Quantech™ fluorometer (Cole Parmer), excitation at 540 nm and emission at 585 nm. Paraquat and $CuSO_4$ stresses were monitored across a range of 20–200 μM and 1–10 mM, respectively.

Flow Cytometry—Fluorescence-activated cell sorter (FACS) cytometry analysis was performed using the H_2O_2-activated green fluorescent dye H_2DCFDA (Molecular Probes). *chrR*$^+$, wild type, and *chrR* mutant cells were grown in LB at 37 °C to mid-exponential phase (A_{660} 0.4–0.7), and then each was split into two cultures (one control and one amended with 1 mM H_2O_2) and incubated as described above for 1 h. Cells (0.5 ml/culture A_{660}) were pelleted by centrifugation, washed once in phosphate-buffered saline, and resuspended in LB containing 20 μM H_2DCFDA, and then incubated for 15 min and diluted 1:500 in phosphate-buffered saline. The fluorescence levels (excitation 488 nm and emission 530 nm) of 50,000 cells were then counted for each strain under each condition using a FACScalibur cytometer (BD Biosciences), as described previously (17). CELLQUEST software (BD Biosciences) was used for data analysis and generation of histograms.

Detection of Protein Carbonylation by Slot-blotting—*chrR*$^+$, wild type, and *chrR* mutant cells were grown in LB at 37 °C to mid-exponential phase (A_{660} 0.4–0.5). 1 ml of cells were removed from each culture and pelleted by centrifugation, and 3 mM H_2O_2 was added to the remaining cultures. Further 1-ml samples were collected at 15 and 60 min. Immediately following centrifugation, each cell pellet was resuspended in 150 μl of lysis buffer (200 mM sodium phosphate, pH 6.5, containing 1% SDS), vortexed vigorously, and boiled for 5 min. The protein concentration of each sample was measured using the Bio-Rad Dc protein assay kit, following which, samples were diluted in lysis buffer to 100 $\mu g \cdot ml^{-1}$ total protein concentration, and 1 volume of 12% SDS was added to each. 10-μl samples were derivatized with 2,4-dinitrophenylhydrazine and neutralized according to the methods of Levine *et al.* (18) and slot-blotted onto polyvinylidene difluoride membranes. The membranes were blocked in 5% nonfat milk powder in TBST (Tris-buffered saline, pH 7.4, plus 0.05% Tween 20) and then incubated for 1 h in 2% nonfat milk powder in TBST containing a 1:500 dilution of rabbit anti-2,4-dinitrophenol or 2,4-dinitrophenyl antibody (DakoCytomation), washed in TBST, and incubated as before with a 1:12,000 dilution of goat anti-rabbit horseradish peroxidase secondary antibody (Sigma). Bands were visualized with the ECL Plus Western blotting kit (Amersham Bisociences) and quantified using TinyQuant© software (Norman Iscove, University Health Network, Toronto, Canada).

Determination of chrR Expression Profile by Quantitative Real-time Reverse Transcription PCR—An overnight culture of wild type *P. putida* KT2440 was used to inoculate two fresh LB cultures, one unamended and one with 1 mM H_2O_2, to a starting A_{660} of 0.1. The cultures were grown at 37 °C with aeration, and (0.5 ml/culture A_{660}) sample volumes were collected from each culture every 2 h, treated with a 1/10 volume of stop solution (5% buffer-saturated phenol in 100% ethanol), mixed by inversion, pelleted at 11,000 revolutions/min for 5 min, and then snap-frozen in a dry ice/ethanol water bath and stored at −80 °C. Total RNA was prepared from these samples by TRIzol® (Invitrogen) extraction followed by the addition of an equal volume of 70% ethanol and purification on an RNeasy column (Qiagen) with on-column DNase treatment. Complete DNA removal in each sample was confirmed by PCR using the *chrR* primers and conditions described below; if product was detected, the samples were treated with RNase-free DNase (Invitrogen) and re-purified with an RNeasy column. Final RNA yield was quantified by A_{260} measurements. Reverse transcription was performed on 0.5 μg of total RNA from each sample using the Qiagen Omniscript reverse transcription kit (Qiagen), and a reverse *chrR* primer (5′-GACGATCTTCAGCGCAAGG-3′). Primers for quantitative real-time reverse transcription PCR were designed (Beacon Designer software package; Premier Biosoft Intl.) to amplify a 118-bp internal *chrR* fragment (*ChrR*2F, 5′-TGAGCCAGGTGTATTCGGTAG-3′; *ChrR*120R, 5′-GACGATCTTCAGCGCAAGG-3′), and quantitative real-time reverse transcription PCR was performed using an iCycler iQ real-time detection system (Bio-Rad) with the Qiagen QuantiTect Sybr® Green PCR kit. Reaction mixtures (final volume, 20 μl) contained 1× QuantiTect MasterMix, 6 pmol of each primer, 1 μl from the corresponding reverse transcription reaction, and DNase/RNase free water. Following hot start (95 °C for 15 min), 40 cycles of 95 °C for 15 s, 54 °C for 30 s, and 72 °C for 20 s were performed. A data acquisition step (83 °C for 10 s) was used and set above the T_m of potential primer dimers to minimize any Sybr Green absorbance due to the latter. Melt

TABLE II
Kinetics of quinone reduction of ChrR

Substrate	V_{max}[b]	K_m[b]	k_{cat}[c]	k_{cat}/K_m	Optimal T[d]	Optimal pH[e]
	$\mu mol \cdot min^{-1} \cdot mg^{-1}$	μM	s^{-1}	$M^{-1} \cdot s^{-1}$	°C	
Benzoquinone	1,400	110	930	8.5×10^6	70	7.5
Menadione	660	230	440	1.9×10^6	60	8.0
Duroquinone	37	210	25	1.2×10^5	70	8.0
CoQ_1	9.6	120	6.4	5.3×10^4	60	8.5
Chromate[a]	8.8	260	5.8	2.2×10^4	70	5.0

[a] As reported in Ref. 3.
[b] Determined at optimal T and optimal pH for each substrate.
[c] Based on a dimeric enzyme molecular mass of 50 kDa (3).
[d] ChrR tolerated a broad range of temperatures, with $V > 60\%$ V_{max} from 20 to 70 °C for all substrates.
[e] ChrR was also very pH tolerant; $V > 70\%$ V_{max} from pH 5.8 to 8.9 for all quinone substrates.

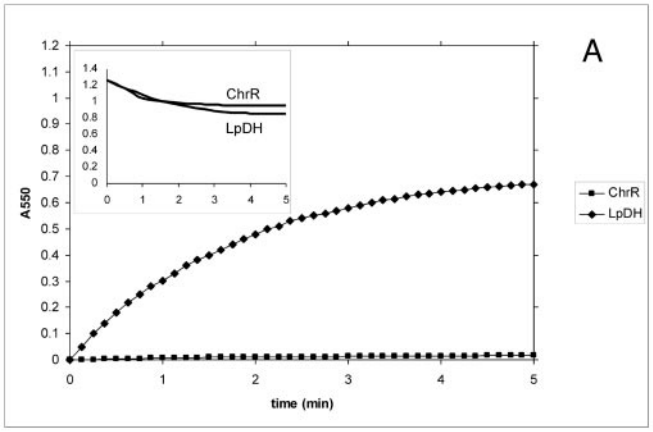

FIG. 1. *A*, reduction of cytochrome *c* monitored spectrophotometrically at 550 nm during LpDH- and ChrR-catalyzed reduction of 50 μM benzoquinone. The appearance of reduced cytochrome *c* during the LpDH-catalyzed reaction indicates at least partial one-electron transfer, whereas the lack of this species in the ChrR-catalyzed reaction signifies a divalent mode of quinone reduction. *Inset*, during these reactions, ChrR oxidized 51 μM NADH, whereas LpDH oxidized 67 μM NADH. *Inset*, *y*-axis shows A_{340}, and *x*-axis shows time in minutes. *B*, addition of ChrR to an LpDH-catalyzed reduction of 50 μM benzoquinone, at the point marked by *arrow 1*, rapidly arrested the reduction of cytochrome *c* relative to LpDH alone (*dashed line*). The addition of fresh 50 μM benzoquinone (*arrows 2* and *3*) re-initiated cytochrome *c* reduction, but with ChrR now present, only 20% as much cytochrome *c* was reduced/unit of benzoquinone.

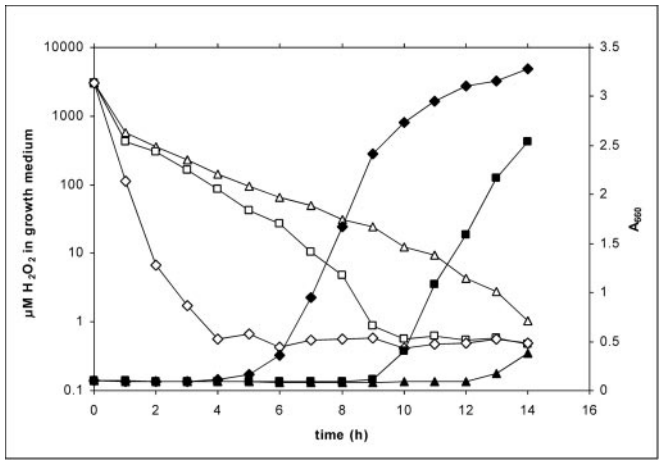

FIG. 2. **H_2O_2-scavenging (*open symbols*) and growth (as measured by A_{660}, *solid symbols*) of *chrR*$^+$ (♦), wild type (■), and *chrR* mutant (▲) cultures inoculated to initial A_{660} 0.1 in LB amended with 3 mM H_2O_2.** This experiment was repeated in triplicate, with consistent results. The figure presents representative data from one of the replicates.

curve analyses displayed a single peak at 87.5 °C, indicating specific *chrR* amplification. The *chrR* copy number in each sample was determined by comparing cycle thresholds to a standard curve of identical reaction mixtures run in parallel, using the PCR-generated full-length *chrR* gene (in the range of 10^3–10^8 copies) as the template.

RESULTS

Kinetic Analysis of Quinone Reduction—Consistent with our proposal that quinone, rather than chromate, reduction may be the primary biological role of ChrR, we showed that this enzyme has a much greater affinity for a variety of quinone substrates (Table II), with a k_{cat}/K_m of 8.5×10^6, 1.9×10^6, 1.2×10^5, and 5.3×10^4 M^{-1} s^{-1} for benzoquinone, menadione, duroquinone, and coenzyme Q_1 respectively, compared with 2.2×10^4 M^{-1} s^{-1} for chromate. The decrease in activity observed with these quinones correlates with their decreasing solubility in water.

Steady state kinetic analyses using menadione as an electron acceptor at various fixed concentrations of NADH (50, 100, or 250 μM) gave parallel lines in double reciprocal plots (not shown). This indicates that ChrR reduces quinones with bi-bi ping-pong (double-displacement) kinetics in two distinct steps: first, a complete reduction of ChrR flavin mononucleotide by NADH, and second, transfer of these two electrons from flavin mononucleotide to the quinone substrate.

Mode of Quinone Reduction—Flavoenzymes can reduce quinones by single-electron, mixed single- and two-electron, and exclusive two-electron transfers (19). The former two mechanisms generate semiquinone intermediates, which in turn are highly prone to generate superoxide radicals (20). The mechanism by which ChrR reduces quinones is therefore of interest, as simultaneous two-electron transfer from the subunit flavin mononucleotide cofactor might suggest a protective role for the enzyme in guarding against formation of reactive semiquinone intermediates. We tested the mechanism of quinone reduction using benzoquinone as a substrate in the presence of a cytochrome *c* trap, because cytochrome *c* is readily reduced both by benzosemiquinone and the superoxide it can generate but not by the fully reduced benzoquinol (21). We found that no cyto-

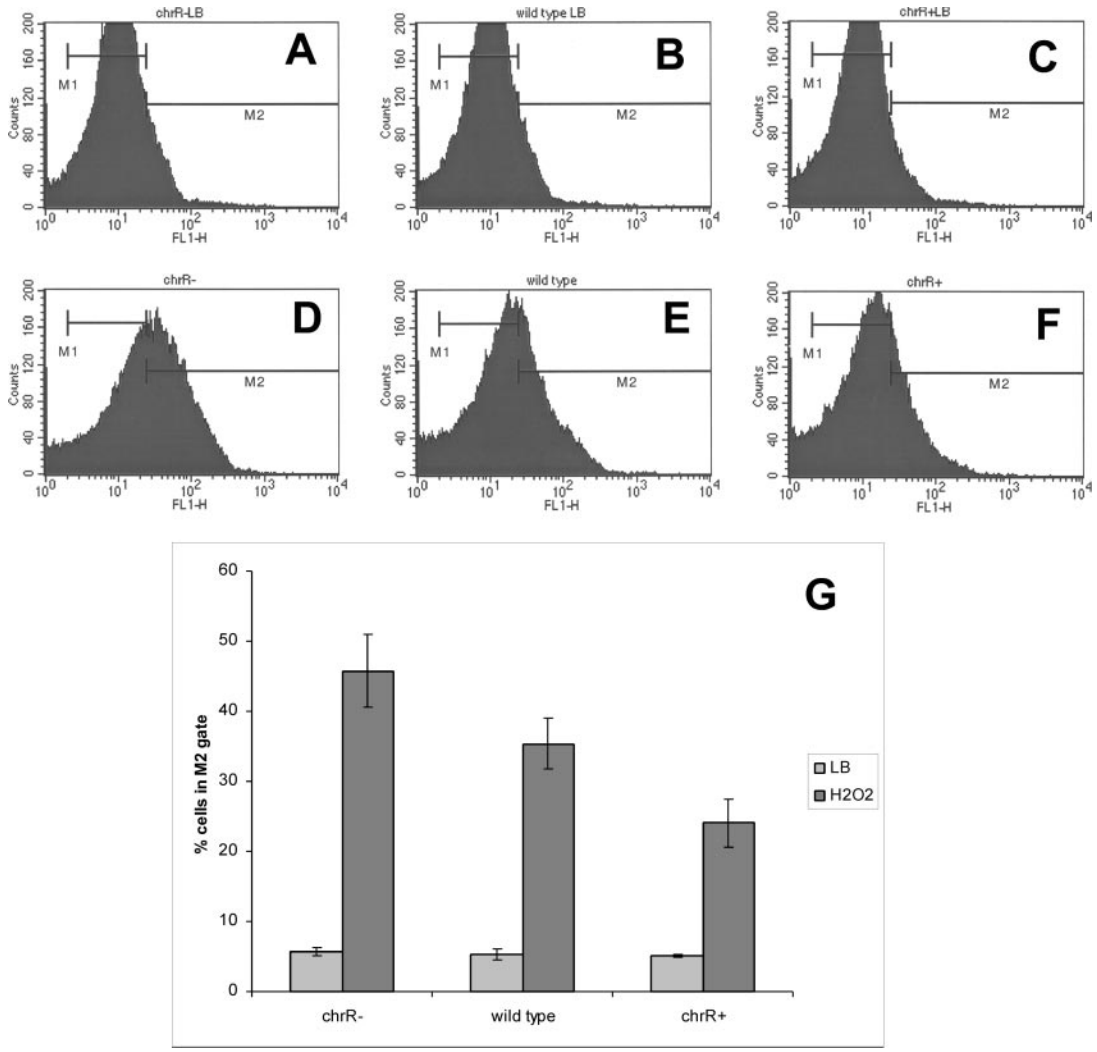

FIG. 3. *A–F*, representative histograms plotting the fluorescence of 50,000 cells treated with 20 μM H_2DCFDA. The *lower boundary* of the M1 gate defines the cut-off for an event to be registered as cellular fluorescence, whereas the M2 gate was established to measure population shifts and delineates approximately the *upper* 5% *fluorescence boundary* of unstressed wild type cells. *A*, unchallenged *chrR* mutant, M2 = (5.7 ± 0.6)%. *B*, unchallenged wild type, M2 = (5.3 ± 0.8)%. *C*, unchallenged *chrR*$^+$, M2 = (5.1 ± 0.2)%. *D*, H_2O_2 (1 mM for 1 h)-challenged *chrR* mutant, M2 = (46 ± 5.2)%. *E*, H_2O_2-challenged wild type, M2 = (35 ± 3.6)%. *F*, H_2O_2-challenged *chrR*$^+$, M2 = (24 ± 3.5)%. *G*, *bar graph* summarizing the percentage of cells within the M2 gate for each strain under each condition. FACS data were collected in triplicate. *Error bars* are mean ± S.E. of 1.

chrome *c* was reduced during ChrR-catalyzed reduction of benzoquinone (Fig. 1*A*), indicating a simultaneous two-electron transfer that avoids formation of a benzosemiquinone intermediate. In contrast, several other bacterial flavoenzymes perform a one-electron reduction of quinones, including LpDH (22). During LpDH-catalyzed reduction of benzoquinone, high levels of cytochrome *c* reduction were observed (Fig. 1*A*). LpDH also oxidized 30% more NADH than ChrR, indicating a drain of electrons via cytochrome *c* reduction (Fig. 1*A*, *inset*).

The above result suggested that ChrR might be able to guard against semiquinone formation by diverting oxidized quinones away from the one-electron reducers. To test this, we added equivalent enzyme units of ChrR to a LpDH-catalyzed reduction of limiting benzoquinone in the presence of cytochrome *c* and found that cytochrome *c* reduction was swiftly halted (Fig. 1*B*, *arrow 1*). Addition of further oxidized benzoquinone to the reaction mix re-initiated cytochrome *c* reduction, but this was likewise soon halted (Fig. 1*B*, *arrows 2* and *3*), and the overall levels of semiquinone formation/benzoquinone reduced were only ~20% of those observed with LpDH alone. This observation provides *in vitro* support for the hypothesis that two-electron reduction by ChrR makes quinones unavailable for one-electron-reducing enzymes, and thereby minimizes formation of reactive semiquinones.

ChrR Confers Resistance to H_2O_2—NQO1 also reduces quinones divalently, and by doing so, protects against oxidative stress in two ways. The first is by guarding against semiquinone formation, and the second is by generating reduced quinols that can quench reactive oxygen species (14). To test the hypothesis that ChrR may play a similar role in *P. putida* in antioxidant defense, we examined the relative ability of *chrR*$^+$-overexpressing, wild type, and *chrR* mutant cells to grow in the presence of exogenous H_2O_2. The different cell cultures exhibited lag phases of varying duration, following which normal sigmoidal growth curves were observed (Fig. 2). The *chrR*$^+$ strain was always the first to recover, followed by the wild type, and finally the *chrR* mutant; in triplicate experiments using 3 mM H_2O_2, the *chrR*$^+$ strain recovered after 5.7 ± 0.6 h, the wild type after 8.7 ± 1.5 h, and the *chrR* mutant after 12.3 ± 2 h. The recovery time correlated with the ability of each strain to remove H_2O_2 from the growth medium, with the lag phase ending ~2 h after the extracellular H_2O_2 concentration had dropped below 5 μM (Fig. 2).

Cultures of *chrR*$^+$-overexpressing, wild type, and *chrR* mu-

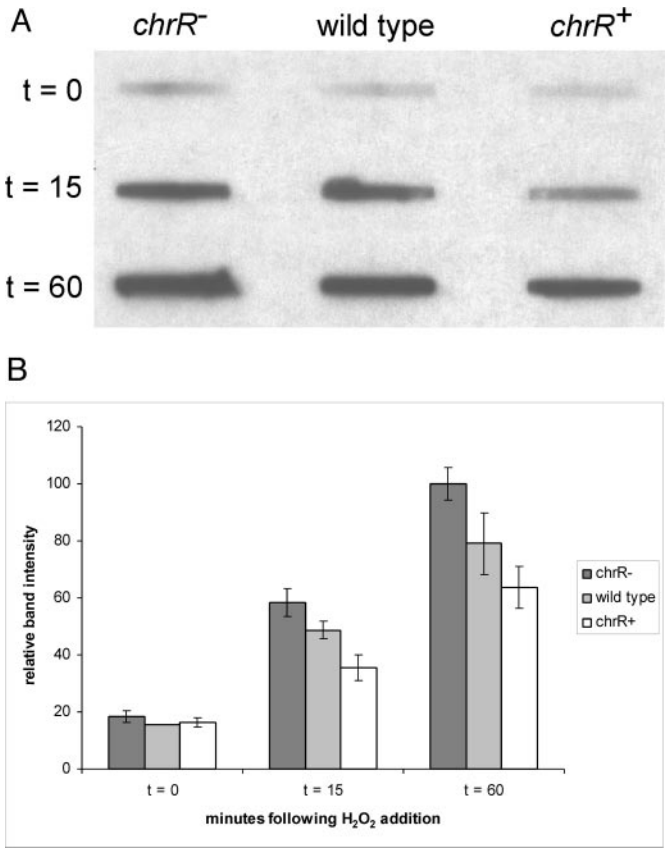

FIG. 4. **Slot-blot analysis of carbonyl levels in unstressed (T = 0 min) and H_2O_2-challenged (3 mM; T = 15, 60 min) cells.** *A*, representative blot indicating accumulation of protein carbonyl groups in H_2O_2-challenged cells; *chrR* mutant > wild type > *chrR*$^+$. *B*, *bar graph* quantifying relative band intensities from the three strains at the three time points; the data are the standardized mean values from three separate experiments (standardized against the intensity of the unstressed (T = 0 min) wild type band), and *error bars* are mean ± S.E. of 1.

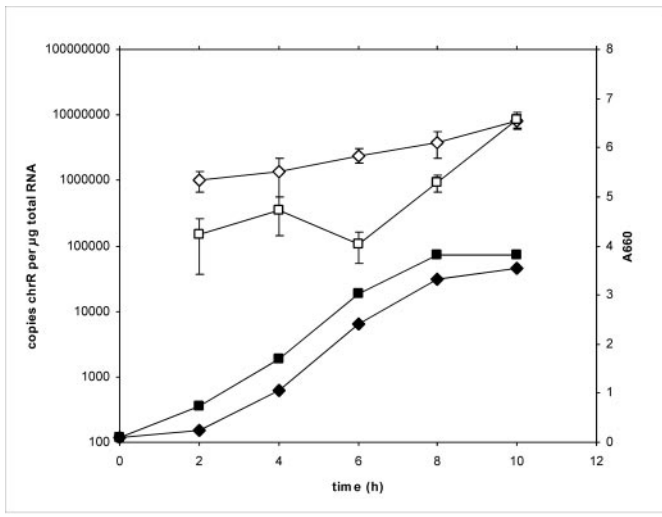

FIG. 5. **A_{660} (*solid symbols*) and *chrR* copy number (*open symbols*) for *P. putida* KT2440 wild type grown in LB (■) and LB amended with 1 mM H_2O_2 (♦).** The LB-only transcript levels were determined from the average of nine separate quantitative real-time reverse transcription PCR reactions, the H_2O_2-amended transcript levels were the average of six reactions, and *error bars* are mean ± S.E. of 1.

tant cells that were grown in parallel but challenged with paraquat or $CuSO_4$ instead of H_2O_2 showed only small variations from one another in their growth kinetics (not shown). This suggests that the two-electron-reduced quinols formed by ChrR are not directly protective against one-electron oxidizers, presumably because quenching of one-electron-oxidized species would in turn generate reactive semiquinones.

ChrR Minimizes Intracellular H_2O_2 Stress—To examine levels of intracellular stress when the *chrR*$^+$, wild type, and *chrR* mutant strains were challenged with H_2O_2, two separate methods were employed. The first of these was FACS cell counting, using the H_2O_2-activated green fluorescent dye dihydrodichlorofluorescein diacetate (H_2DCFDA). This dye has previously been used to measure H_2O_2 in eukaryotic cells; we found it effective for FACS in bacteria as well. Mid-exponential phase cells were grown for 1 h in H_2O_2-amended or control medium, washed in phosphate-buffered saline, incubated for 15 min in LB containing H_2DCFDA, and then the fluorescence levels of 50,000 cells were counted for each strain under each condition. A gate (M2) was established that delineated the upper 5% of fluorescent cells for the unstressed wild type (Fig. 3*B*). Although there was no significant difference between the three strains in the absence of exogenous stress (Fig. 3, *A–C*, *G*), in the presence of H_2O_2, the percentage of gated cells for the *chrR* mutant was 46 ± 5.2, the wild type strain was 35 ± 3.6, and the *chrR*$^+$ strain was 24 ± 3.5 (Fig. 3, *D–G*).

The above data indicated that levels of intracellular H_2O_2 stress were inversely correlated with *chrR* expression. If so, this should be reflected by the protein carbonylation levels in each strain. Carbonyl groups are introduced into protein side chains by site-specific oxidative modifications, and although this is not the sole mechanism of protein carbonyl formation *in vivo*, carbonyl quantification is believed to provide an accurate estimate of the oxidation status of proteins (18, 23). Crude protein extracts were obtained following growth of mid-exponential phase cells for 0 and 15 min and 1 h in H_2O_2-amended medium. These were derivatized with the carbonyl reagent 2,4-dinitrophenylhydrazine, slot-blotted onto a polyvinylidene difluoride membrane, and oxidatively modified proteins were detected with anti-2,4-dinitrophenol or 2,4-dinitrophenyl antibodies. A representative blot is pictured in Fig. 4*A*; as for the FACS data, quantification of immunoassay bands from triplicate blot experiments indicated that, although there was no significant difference between the unchallenged strains, in H_2O_2-treated cells, the levels of stress correlated inversely with the levels of ChrR present, with the *chrR*$^+$-overexpressing strain exhibiting the lowest protein carbonylation levels (Fig. 4*B*).

ChrR Is Induced by H_2O_2—The above experiments suggested that induction of *chrR* expression provides a means for cells to defend themselves against H_2O_2 stress. To examine whether or not wild type cells might employ such a mechanism, we analyzed *chrR* expression by quantitative real-time reverse transcription PCR to see whether transcription of this gene was induced by H_2O_2. *chrR* transcript levels increased even in unstressed cells with progression into stationary phase, but *chrR* was induced much more rapidly in cells challenged with 1 mM H_2O_2 (Fig. 5). During exponential growth, *chrR* transcript levels were ~10-fold higher in the H_2O_2-challenged cells. These results indicate that ChrR is likely an active component of the *P. putida* response to H_2O_2 stress.

DISCUSSION

Two members of the widespread NADH_dh2 family have previously been shown to reduce quinone substrates, NQO1 reductase of *Arabidopsis thaliana* (24) and the ferric reductase FerB of *Paracoccus denitrificans* (13). However, no experimental evidence has hitherto been presented for any biological significance of this activity. Here we demonstrated a clear benefit of the quinone reductase activity of ChrR to *P. putida* in guarding against H_2O_2 stress. The ability of ChrR as a soluble

reductase to reduce a variety of quinone substrates by a divalent ping-pong electron transfer mechanism, and its clear antioxidant role, are properties reminiscent of the mammalian enzyme NQO1 (14). Similar to NQO1, ChrR appears to exercise its antioxidant effects in two ways: indirectly, by diverting oxidized quinones away from cellular one-electron reducers and thereby avoiding generation of reactive semiquinones, and directly, through quinone-mediated quenching of H_2O_2. It should be noted that the *P. putida* KT2440 genome (25) encodes a further putative quinone oxidoreductase (GenBank™ accession number AAN69317), which shares 36% amino acid identity with mammalian NQO1, and it may be that there is some functional overlap between this enzyme and ChrR. Nonetheless, the clear phenotypic differences between the H_2O_2-challenged *chrR*$^+$, wild type, and *chrR* mutant strains, supported by the strong induction of wild type *chrR* both in stationary phase and by H_2O_2, indicate a non-redundant role for ChrR in guarding against H_2O_2 stress.

This is the first time that a soluble bacterial quinone reductase has been shown to contribute to cellular antioxidant defense. This activity might guard both against H_2O_2 generated as a by-product of aerobic respiration and that produced by external factors. As a soil-dwelling, primarily saprophytic organism, *P. putida* is regularly exposed to a wide range of environmental insults. In particular, when plant cells come into contact with bacteria, the plant cells release an immediate (and non-bacterial strain-specific) burst of H_2O_2 (26). In tobacco cell suspensions, this H_2O_2 burst typically falls below the 100 μM range, and Baker *et al.* (27) demonstrate that catalase, because of its high K_m (anywhere from 47 to 1100 mM), is inefficient at scavenging H_2O_2 at such concentrations. Indeed, wild type *P. putida* were no better able to survive a range of H_2O_2 concentration from 0.001 to 1 mM than an isogenic catalase-deficient mutant (27). This observation highlights a need for one or more alternative mechanisms for H_2O_2 scavenging in *P. putida*, and we propose that quinone-mediated quenching by ChrR is likely to be particularly relevant at μM levels of H_2O_2. This hypothesis is strongly supported by the H_2O_2-scavenging profiles of the *chrR*$^+$, wild type, and *chrR* mutant strains presented in Fig. 2. High levels of ChrR give a clear advantage to the overexpressing strain at all H_2O_2 concentrations, but a phenotypic difference between the wild type and *chrR* mutant is largely masked until the concentration of H_2O_2 in the growth medium falls below ~100 μM. This is presumably because catalase is effective at scavenging H_2O_2 at higher concentrations, but at concentrations well below its K_m, the contribution of this enzyme diminishes, and the advantage conferred to the wild type by ChrR becomes clear.

ChrR may also contribute to the survival of *P. putida* in the environment by preventing redox cycling of soluble quinones. Not only are quinonoid compounds with pro-oxidant capacity generated internally from the metabolism of aromatics, there are also many external quinone sources. In particular, plants secrete quinones, such as plumbagin and juglone, as defensive agents or to gain a competitive advantage (28). Solubility of these quinones is promoted by their lack of isoprenoid side chains, and their redox cycling imposes severe oxidative stress; indeed, plumbagin and juglone are commonly used to induce oxidative stress in experimental studies (28). As we have shown here, the ability of ChrR to carry out divalent quinone reduction would divert these species away from one-electron reducers within a cell and limit their pro-oxidant activity.

Our ongoing work is aimed at enhancing ChrR activity to promote chromate bioremediation. Previously, we have shown that enzymatic chromate reduction is unavoidably associated with H_2O_2 generation (3). Despite this, the *chrR* mutant was impaired relative to wild type, not only in chromate transformation rate but also in viability in the presence of chromate (3). We speculated, then, that the protective effect of ChrR might stem from an ability to pre-empt one-electron reducers from carrying out a partial reduction of Cr(VI) to the redox-cycling species Cr(V) and subsequently provided *in vitro* experimental evidence supporting this hypothesis (4). However, in light of the data presented here, it now seems likely that the quinone-mediated antioxidant activity of ChrR was also a significant factor contributing to the superior viability of the wild type cells. Thus, enhancing ChrR activity may not merely increase rates of chromate transformation, it may also promote the ability of remediating cells to withstand an associated increase in oxidative burden.

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