J. Microbiol. Biotechnol. (2007), **17**(8), 1344–1352

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Identification of *Pseudomonas aeruginosa* Genes Crucial for Hydrogen Peroxide Resistance

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Received: March 31, 2007 Accepted: April 28, 2007

Abstract An opportunistic human pathogen, *Pseudomonas aeruginosa*, contains the major catalase KatA, which is required to cope with oxidative and osmotic stresses. As an attempt to uncover the H_2O_2-dependent regulatory mechanism delineating *katA* gene expression, four prototrophic H_2O_2-sensitive mutants were isolated from about 1,500 Tn*phoA* mutant clones of *P. aeruginosa* strain PA14. Arbitrary PCR and direct cloning of the transposon insertion sites revealed that one insertion is located within the *katA* coding region and two are within the coding region of *oxyR*, which is responsible for transcriptional activation of several antioxidant enzyme genes in response to oxidative challenges. The fourth insertion was within PA3815 (IscR), which encodes a homolog of the *Escherichia coli* iron-sulfur assembly regulator, IscR. The levels of catalase and SOD activities were significantly reduced in the *iscR* mutant, but not in the *oxyR* mutant, during the normal planktonic culture conditions. These results suggest that both IscR and OxyR are required for the optimal resistance to H_2O_2, which involves the expression of multiple antioxidant enzymes including KatA.

Keywords: *Pseudomonas aeruginosa*, KatA, OxyR, IscR, H_2O_2, catalase, SOD

Virtually all aerobic and facultatively aerobic organisms come into contact with reactive oxygen species (ROS) such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^-), which are inevitably generated as a result of normal metabolic processes [10, 18]. To counter the destructive nature of ROS, such organisms have evolved antioxidant defense mechanisms involving a set of regulatory systems that sense ROS-derived signals and transduce the signals into expression of target genes whose gene products are involved in various processes to reduce the harmful effect of the ROS [45].

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The regulatory networks as well as the effector proteins in response to oxidative stresses have been best characterized in enteric bacteria such as *Escherichia coli* and *Salmonella typhimurium* [7]. These bacteria exert distinct responses against H_2O_2 and O_2^- [7], and the major response to H_2O_2 is governed by a 34-kDa LysR-type transcription factor, OxyR.

E. coli OxyR acts as both the sensor and the transactivator in response to H_2O_2 [6]. At least nine genes are induced by H_2O_2 treatment, which requires the presence of functional OxyR [39]. OxyR is activated by disulfide bond formation between two cysteine residues (C199 and C208), which is common in redox-active cysteine-containing regulatory proteins such as OhrR and Hsp33 [1, 24] and positively regulates the expression of a small regulatory antimutator RNA encoded by *oxyS*, the ferric uptake regulator (Fur), the major bifunctional catalase-peroxidase (HPI encoded by *katG*), alkyl hydroperoxide reductase (AhpCF), glutathione reductase (GorA), a nonspecific DNA-binding protein (Dps), and glutaredoxin 1 (GrxA). Each of these proteins is important in coping with H_2O_2-mediated stressful conditions, and GrxA is the primary reducing factor maintaining the redox potential in the cytoplasm (−185 mV), which rapidly reduces the C199-C208 disulfide bond and thus deactivates OxyR whose redox potential is about −180 mV [46]. This rapid regeneration of reduced OxyR accounts for the autoregulatory loop of the OxyR activation mechanism. OxyR also acts as a repressor of its own expression, similar to the other LysR family of transcriptional regulators, which is not associated with its redox state [34].

The detoxification of the harmful effects exerted by H_2O_2 involving similar regulatory mechanisms may be crucial, especially for bacterial pathogens, because they face exposure to exogenous H_2O_2 and related ROS, which are generated at high millimolar levels within the phagosomal vacuole, during their infection processes [11]. *Pseudomonas aeruginosa* is one of the important model bacteria, which is an opportunistic human pathogen primarily causing fatal infections in immunocompromised individuals such as

hospitalized patients and those suffering from severe burns or other traumatic skin damage or from cystic fibrosis [3]. This ubiquitous gammaproteobacterium deploys an arsenal of diverse virulence factors to intoxicate human hosts as well as diverse nonmammalian hosts that include plants, nematodes, insects, and slime molds [15, 21, 31]. It also kills the mycelial form of the dimorphic fungus *Candida albicans*, and a Gram-positive bacterium, *Bacillus subtilis*, which requires some shared subsets of virulence factors such as Las and PQS quorum sensing systems [15, 30].

Although *P. aeruginosa* OxyR is recently reported to be an important virulence factor in fly and rodent models [19], much remains to be discovered about the regulatory networks involved in adaptive response to H_2O_2 and the virulence mechanism associated with them. In contrast to the case for *E. coli* as well as several other proteobacteria [16, 37], the *P. aeruginosa* OxyR regulon appears not to include the major catalase KatA [27], which remains constitutively high during the normal aerobic growth [4]. Instead, genes under the direct OxyR control in *P. aeruginosa* include a second monofunctional catalase (*katB*) and two alkyl hydroperoxide reductases (*ahpB* and *ahpCF*), all of which are dramatically increased upon exposure to H_2O_2 [4, 27].

In our previous study, the major catalase KatA was found to be critical for the resistance to H_2O_2 and osmotic stresses, the adaptive response to H_2O_2 and full virulence [20], suggesting that redox-responsive regulatory systems might be required for the *katA* gene expression upon H_2O_2 treatment. Based on this and the fact that the redox-dependent regulators and enzymes of *P. aeruginosa* are important in its survival and/or virulence in host tissues [9, 20, 44], we decided to identify the genes that are important in H_2O_2 resistance. In the present study, we report the isolation and characterization of the mutants identified from a subset of Tn*phoA* random transposant clones of *P. aeruginosa* strain PA14, whose growth is impaired in the presence of H_2O_2. As a result, IscR was newly identified as affecting the level of catalase and superoxide dismutase activities during the normal growth conditions of this bacterium.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Strains and plasmids used in this study are listed in Table 1. *P. aeruginosa* PA14 was used for Tn*phoA* transposon mutagenesis [22] and isolation of H_2O_2-sensitive mutants. *E. coli* S17-1 containing pRT733 [40] was used for transposon delivery *via* conjugal transfer. All bacterial cells were grown in LB or M63 minimal media for liquid culture or on LB agar for plate culture as described previously [21, 23].

Transposon Mutagenesis

P. aeruginosa PA14 was mutagenized using plasmid pRT733 carrying the Tn*5*-derived transposon Tn*phoA* described previously [32], but with the following modifications: the recipient PA14 cells and the donor *E. coli* S17-1 carrying pRT733 cells were grown in LB broth for 12 h at 42°C and 37°C, respectively. Donor and recipient cells were plated together on LB agar plates and incubated at 37°C for 20 h,

Table 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Strain name and relevant characteristics[a]	Reference
P. aeruginosa strains		
PA14	Wild-type laboratory strain; Rif^R	[31]
iscR	PA14 Δ*iscR*; Rif^R	This study
oxyR	PA14 Δ*oxyR*; Rif^R	This study
katA	PA14 Δ*katA*; Rif^R	[20]
HS1	PA14 Tn*phoA* insertion mutant in *katA*; Rif^R, Km^R	This study
HS2	PA14 Tn*phoA* insertion mutant in *oxyR*; Rif^R, Km^R	This study
HS3	PA14 Tn*phoA* insertion mutant in *iscR*; Rif^R, Km^R	This study
HS4	PA14 Tn*phoA* insertion mutant in *oxyR*; Rif^R, Km^R	This study
E. coli strains		
S17-1	RP4-2-Tc::Mu-Km::Tn*7*, *pro* (r^- m^+) *Mob* Tp^RSm^R	[38]
DH5α	F2 f80d*lacZ* ΔM15 Δ(*lacZYA-argF*) U169 *endA1 recA1 hsdR17 deoR gyrA96 thi-1 relA1 supE44*	[33]
Plasmids		
pRT733	Tn*phoA* delivery plasmid; Km^R	[22]
pEX18T	Positive selection suicide vector; Ap^R, Cb^R	[15]
pJN105	pBRR-1MCS5, *araC-*P_{BAD}; Gm^R	[26]
pUCP18	Multicopy plasmid; Ap^R, Cb^R	[36]

[a]Rif^R, rifampin-resistant; Km^R, kanamycin-resistant; Tp^R, thrimethoprim-resistant; Sm^R, streptomycin-resistant; Ap^R, ampicillin-resistant; Cb^R, carbenicillin-resistant; Gm^R, gentamycin-resistant.

and PA14 cells carrying a chromosomal insertion of the transposable element were selected on LB agar containing rifampicin (150 μg/ml) (to counterselect the *E. coli* donor cells) and kanamycin (Km, 500 μg/ml) (to select for Tn*phoA*-containing *P. aeruginosa* cells). Single colonies were patched to a selective master plate and the well-growing colonies were subjected to the confirmation of Tn*phoA* transposition by PCR amplification of a 784-bp fragment from the Km marker of Tn*5* using a primer set Km-F (5'-GCA TGA TTG AAC AAG ATG G-3') and Km-R (5'-TCA AGA AGG CGA TAG AAG G-3'). About 1,500 transposants were subjected to this screen.

Stress Treatment in Liquid Culture and Screening for H_2O_2-sensitive Mutants

Cells were grown in LB broth (3 ml) containing various amounts of H_2O_2 (0.3 to 10 mM) and the growth inhibition was monitored by optical density measurement at 600 nm (OD_{600}). For primary screening, each Tn*phoA* transposant clone that had been grown in a 96-well plate-based LB broth (100 μl) was transferred (1% v/v) into a 96-well plate-based fresh LB broth (100 μl) that contained 800 μM H_2O_2. Cells were grown for 8 h and the growth impairment was monitored based on OD_{600} measurement using a SPECTRA Max 250 ELISA reader (Molecular Devices Corp). Sixteen non-growing or reduced growing clones, whose OD_{600} was less than 0.05, were initially obtained. Eight of them with slight growth defect on M63 minimal media [21] containing 10 mM citrate as a carbon source were excluded and the remaining 8 clones were subjected to the secondary screening by spotting assays on solid agar plates. After verification of the H_2O_2 sensitivity, four clones (HS1 to 4) were selected as H_2O_2-sensitive mutants from this screen.

Stress Treatment on Plate Culture

Disc diffusion assay and spotting assay were performed as described previously [12, 13, 20, 43]. For disc diffusion, cells were grown in LB broth at 37°C to OD_{600} of 1.0 and cell lawns were generated by overlaying LB agar plates with soft LB agar (0.7%) containing about 10^8 cells. Following 1 h air drying, 3 μl droplets of H_2O_2 (8.8 M), menadione (2 M), cumene hydroperoxide (5 M), and ferrous chloride (1 M) were spotted on the filter discs placed on the cell plates. For spotting assay, cells were grown in LB broth at 37°C to an OD_{600} of 1.0. Ten-fold serial dilutions of the cells in LB broth (3 μl) were spotted onto an LB agar medium containing 100 μM H_2O_2.

Identification of Transposon Insertion Sites

Two methods (arbitrary PCR and direct cloning) followed by sequencing are used to determine the transposon insertion sites. Arbitrary PCR was performed according to Okura *et al.* [28]. Briefly, the oligonucleotide primer pairs Tn5Ext (5'-GAA CGT TAC CAT GTT AGG AGG TC-3') and either Arb1 (5'-GGC CAC GCG TCG ACT AGT CAN NNN NNN NNN GAT ATA-3') or Arb1A (5'-GGC CAC GCG TCG ACT AGT ANN NNN NNN NNG TAT A-3') were used in the first PCR, whose products were used as the template for the second PCR using the oligonucleotides Arb2 (5'-GGC CAC GCG TCG ACT AGT AC-3') and Tn5Int (5'-CGG GAA AGG TTC CGT TCA GGA CGC-3'). The most prominent band from the second PCR was purified and sequenced. For direct cloning, a pEX18T-derivative containing the *tnpA* segment was used to target the right end of the Tn*phoA* transposon of the Tn*phoA* mutant chromosomes. After single-crossover recombination, the chromosomal DNAs were isolated from the cointegrates of each Tn*phoA* mutant, digested with SnaBI and NruI, and subjected to self-ligation. The circularized replicons were rescued and their nucleotide sequences were determined.

Allelic Exchange

Allelic exchange to transfer the Tn*phoA* insertion region to a new *P. aeruginosa* background was performed according to Choi *et al.* [5] with slight modification. Cells from overnight stationary-phase cultures grown in LB were harvested for 2 min at 8,000 rpm. The cell pellet was washed twice with 1 ml of 300 mM sucrose and then resuspended in 300 mM sucrose, and used for electroporation. Chromosomal DNA samples (500 ng) obtained from the Tn*phoA* mutants were mixed with the electrocompetent cells. After applying a pulse (25 μF, 2.5 kV/cm, 5 msec), 1 ml of LB medium was added, and the cells were transferred to a glass tube and shaken for 1 h at 37°C for regeneration. Cells were spread on LB plates containing Km (200 μg/ml) and incubated at 37°C, until colonies appeared.

Gene Disruption and Complementation

The in-frame deletion mutants for *oxyR* and *iscR* were created using pEX18T [14]. The oligonucleotide primers were designed based on the sequences from *P. aeruginosa* strain PA14 [25]. For the *oxyR* mutant, a 1.6-kb fragment encompassing the *oxyR* gene was amplified from the PA14 chromosome using the oligonucleotide primers oxyR_N1 (5'-CCG GAA TTC GCC TGG GAA AGC G-3') and oxyR_C1 (5'-TGA ATT CGT CTC CTT CCT ACA AC-3'; underline denotes the engineered BamHI site). The PCR product was cloned and used for the template for inverse PCR using the oligonucleotide primers oxyR_DN (5'-GAC CAT GGG CCA CAT GGT CGC C-3') and oxyR_UC (5'-GCC CAT GGG CGT CAG GCG CAC G-3'; underline denotes the engineered NcoI site). The inverse PCR product was digested with NcoI followed by self-ligation to create the in-frame deletion of approximately 55% of the *oxyR* coding region. For *iscR* mutant, 2 oligonucleotide primers

iscR_N1 (5'-CCA GCC GAA TTC GTG GGA ACG CG-3') and iscR_C1 (5'-CGC CGA ATT CAA GGA TGA GGA CG-3'; underline denotes the engineered BamHI site) were used to amplify the 0.84-kb fragment from the *iscR* gene. The PCR product was cloned and digested with SphI and SalI followed by linker-inserted ligation to create the 206-bp in-frame deletion of approximately 42% of the *iscR* coding region. The linker was created by annealing two short oligonucleotides (TCG AGA CCA TGG TCA TG and ACC ATG GTC; underline denotes the complementary sequences). All double-crossover deletion mutants were obtained by sucrose resistance selection from the single-crossover cointegrates and verified by PCR and mutant phenotypes (*e.g.*, hypersensitivity to H_2O_2).

Complementation experiments were performed using multicopy plasmids, pJN105 [26] and pUCP18 [36]. For OxyR expression, the same 1.6-kb fragment for deletion that covers the *oxyR* coding region was cloned into pJN105. For IscR expression, the same fragment (0.84 kb) for deletion was cloned into pUCP18. These plasmids were introduced into *P. aeruginosa* cells by electroporation [5, 42].

Activity Staining: Catalase and SOD

Catalase activity staining was performed according to the method of Wayne and Diaz [41]. Briefly, cell extracts (20 μg) were applied to a 7% native polyacrylamide gel. After electrophoresis, the gel was washed in distilled water and then placed in 5 mM H_2O_2 for 10 min. The rinsed gel was transferred to a freshly prepared solution of 1% (w/v) ferric chloride and 1% (w/v) potassium ferricyanide. Once green color began to appear on the gel, the reaction was stopped by washing the gel in distilled water. SOD activity staining was performed according to the method of Beauchamp and Fridovich [2]. The cell extracts (20 μg) were applied to a 12% native polyacrylamide gel. After electrophoresis, the gel was stained by incubation in a solution containing 2.5 mM nitroblue tetrazolium for 25 min, followed by incubation in 50 mM potassium phosphate buffer (pH 7.8) containing 28 μM riboflavin and 28 mM tetramethyl ethylene diamine for 20 min in the dark. The gel was placed in distilled water and exposed on a light box for 15 min until a dark-blue background color appeared.

Results and Discussion

Dose-dependent Inhibition of *P. aeruginosa* Growth by H_2O_2

Prior to screening for H_2O_2-sensitive mutants of *P. aeruginosa*, we measured the growth inhibition of *P. aeruginosa* PA14 cells and its isogenic *katA* mutant by

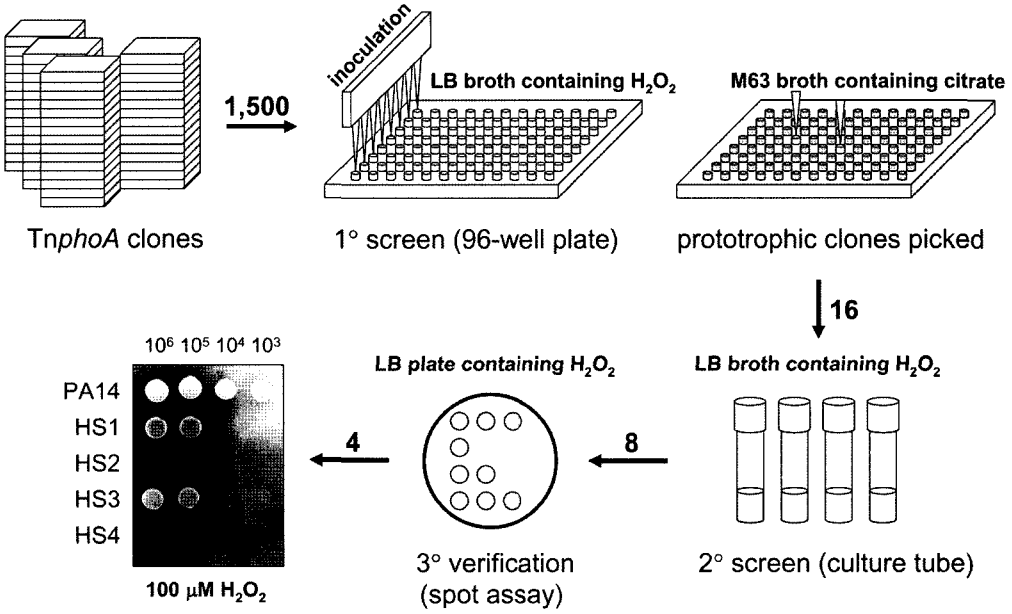

Fig. 1. Schematic representation of H_2O_2-sensitive mutants.

One-thousand five-hundred Tn*phoA* clones were individually inoculated into the 96-well-based fresh culture broth of either LB medium containing 800 μM H_2O_2 for the primary screening or M63 containing 10 mM citrate as the sole carbon source for the prototrophic growth; 8 h later, growth impairment in H_2O_2-LB media was monitored based on OD_{600} comparison with the corresponding growth in citrate-M63 media. Sixteen clones were subjected to the secondary screening using the ordinary test tube-based culture in the presence of 1 mM H_2O_2, after which 8 clones were chosen for the tertiary verification by spotting serially diluted cultures onto an LB agar medium containing 100 μM H_2O_2. The four H_2O_2-sensitive mutants (HS1 to HS4) were finally isolated and four 10-fold dilutions of each mutant cell were spotted onto an LB agar medium containing 100 μM H_2O_2. The numbers (10^6 to 10^2) indicate the CFU of the cell spots.

H_2O_2 treatment in planktonic liquid culture. The growth of PA14 cells was inhibited by H_2O_2 in a dose-dependent manner (data not shown). The drastic growth inhibition was observed by 10 mM H_2O_2, whereas less than 8 mM H_2O_2 allowed cells to grow to an optical density at 600 nm (OD_{600}) of more than 1.5. In contrast to PA14 cells, the growth of *katA* mutant cells was completely inhibited by 0.5 mM H_2O_2 during the liquid culture (data not shown). Thus, we defined the H_2O_2-sensitivity of a clone during the normal aerobic planktonic culture as the inability to grow in LB broth containing 0.5 to 10 mM H_2O_2, depending on the culture conditions.

Isolation of H_2O_2-sensitive Mutants from Tn*phoA* Mutant Clones

To identify *P. aeruginosa* H_2O_2-resistance genes, we designed a high throughput and cost-effective screening procedure to identify bacterial mutants with H_2O_2-sensitivity. As described in Materials and Methods and Fig. 1, a total of approximately 1,500 PA14 Tn*phoA* insertion mutants were individually inoculated in 96-well plate-based LB broth containing 800 μM H_2O_2 and then screened for reduced or impaired growth after 8 h incubation at 37°C. Sixteen candidates for strains with reduced or impaired growth after the primary screening were obtained. To determine whether the observed impairment or reduction in growth was due to a problem of general metabolism and/or growth, or was the result of the disruption of a gene responsible for H_2O_2-resistance, we measured the basal growth parameters in planktonic batch cultures such as doubling time, lag time, and saturation optical density and those of plate cultures using M63-citrate minimal medium such as colony size and time of colony appearance. Based on these, 8 clones were chosen for the further (tertiary) verification, which was based on cell spotting assay as in Materials and Methods. Only four mutant clones (named as HS1 to HS4) were significantly reduced out of the 8 clones; HS2 and HS4 are highly sensitive; HS1 is moderately sensitive, which is similar to the *katA* mutant (data not shown); HS3 is weakly but significantly sensitive to H_2O_2 (Fig. 1).

Each of the 4 mutant clones contained a single transposon insertion as verified by Southern blot analyses (data not shown), where two clones (HS2 and HS4) displayed the same band patterns, indicating that the two mutants were identical (see below). Each of the Tn*phoA* insertions was individually transferred into PA14 by allelic exchange, and the H_2O_2 susceptibility of the resulting recombinant transposants were identical compared with that of the original mutants (data not shown), suggesting that the H_2O_2-sensitivity phenotype of each clone is due to Tn*phoA* insertion.

Molecular Characterization of the H_2O_2-sensitive Mutants

To identify the transposon insertion sites of the isolated mutants, we carried out arbitrary PCR (semi-random PCR) [28] and direct cloning, through which we determined the junction region between the transposon and the genomic DNA of each mutant. As might be expected from the Southern analysis as well as the similar phenotypic characteristics (data not shown; Fig. 1), HS2 and HS4 turned out to be identical, whose transposon insertion was mapped within the *oxyR* gene encoding a peroxide-responsive transcription factor to regulate several antioxidant enzymes (*katB* and *ahpCF*). HS1 contained the Tn*phoA* insertion within the *katA* coding region, accounting for the similar susceptibility

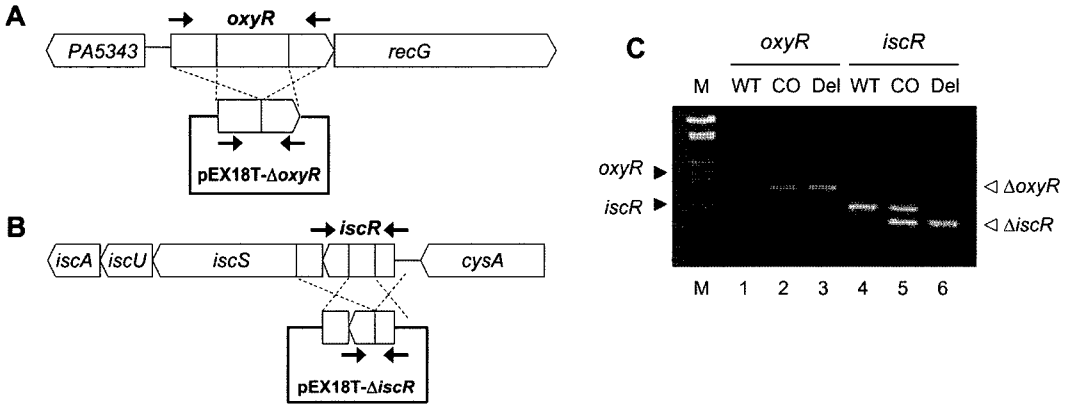

Fig. 2. Creation and verification of deletion mutants.

Based on the PA14 sequences, PCR-based deletions of *oxyR* (PA5344) and *iscR* (PA3815) genes were generated, cloned in pEX18T, and used to create single in-frame deletion mutants in wild-type PA14 (WT), *via sacB*-dependent cointegrate segregation. Schematic representations of deletion and double-crossover schemes for *oyxR* (**A**) and *iscR* (**B**) are shown (not to scale), with the PCR verification of their predicted genetic structures (**C**). The PCR product sizes of the intact genes (designated by the solid arrowhead on the left) for *oxyR* and *iscR* are 1.6 and 0.84 kb, respectively, whereas those of deletions (designated by the empty arrowhead on the right) are 1.1 and 0.6 kb, respectively. The size marker (M) contains approximately 21, 5.1, 4.9, 2.0, 1.8, 1.6, 1.4, 1.0, 0.8, and 0.6 kb DNA fragments generated by EcoRI/HindIII digestion of phage λ DNA (lane M). Abbreviations: WT, wild-type (lanes 1 and 4); CO, cointegrate (lanes 2 and 5); Del, deletion mutant (lanes 3 and 6).

as the *katA* deletion mutant. *oxyR* and *katA* mutants are well known to be highly sensitive to H_2O_2 stresses [20, 27], and the isolation of these mutants indicates the validity of this screening procedure. The last insertion was identified as located within PA3815. The PA3815 gene has not been characterized so far, which encodes a transcription factor closely similar to *E. coli* IscR (75% similarity and 61% identity). *E. coli* IscR is the repressor of the *iscRSUA* operon that acts as the major iron-sulfur cluster assembly system of *E. coli*. *E. coli* has another iron-sulfur cluster assembly system, *sufABCDSE*, whereas the *P. aeruginosa* genome appears to possess no homolog of the *E. coli suf* gene cluster (data not shown). *E. coli* IscR itself contains two iron-sulfur (Fe-S) clusters and acts as its own repressor when it is oxidized [35], and the isolation of its homolog in *P. aeruginosa* as a H_2O_2-sensitive mutant gene is noteworthy. Although it warrants further experimental verification that the PA3815 gene is the IscR ortholog in *P. aeruginosa*, we named PA3815 as IscR, based on two reasons; first, there is no other IscR homolog on the *P. aeruginosa* PAO1 and PA14 genomes; second, the PA3815 gene is within the gene cluster, whose genetic organization is almost identical to the *E. coli iscRSUA* operon (Fig. 2A).

To verify that the gene disruption of *oxyR* and *iscR* caused the hypersensitivity to H_2O_2, we created the in-frame deletion mutants for *oxyR* and *iscR* as described in Materials and Methods. Fig. 2 shows the gene disruption schemes and their genetic verification. The hypersensitivity phenotypes of *oxyR* and *iscR* mutants as well as their corresponding Tn*phoA* insertion mutants (HS2 and HS3, respectively) were fully complemented by introducing the cognate gene segment in a multicopy plasmid, pUCP18 or pJN105 (data not shown). This result suggests that both OxyR and IscR are required for full resistance to H_2O_2, like the major catalase KatA.

Susceptibilities of the H_2O_2-sensitive Mutants to other Stress Treatments

To determine whether the *P. aeruginosa* genes necessary for H_2O_2 resistance are also required for resistance in related stresses, the *katA*, *oxyR*, and *iscR* mutants were individually tested by disc diffusion assay using a redox-cycling agent, menadione (MD), which generates superoxide radicals in aerobic conditions [8]. These mutants exhibited no less susceptibility to ferrous iron and to cumene hydroperoxide (CHP) compared with the wild-type (Fig. 3). This result suggests that they might have similar expression of the alkyl hydroperoxidase system (AhpB, AhpC, and AhpD). Since it is already known that OxyR is involved in the *ahpCF* gene expression as well as CHP resistance in *P. aeruginosa* strain PAO1 [12], the apparent difference in CHP resistance might be associated with strain variation. In fact, we found that both *katA* mutants of PA14 and PAO1 displayed differential susceptibilities to a superoxide-generating agent, paraquat, and there might be some strain-dependent variations in responses to at least oxidative stress treatments (data not shown). Since the *oxyR* mutant is highly sensitive to MD (Fig. 3C), it needs to be further evaluated whether this susceptibility is the elevation of intracellular reactive oxygen species such as H_2O_2, which requires the presence of oxygen and the superoxide dismutases (SODs) as well to convert the generated superoxide radical to H_2O_2.

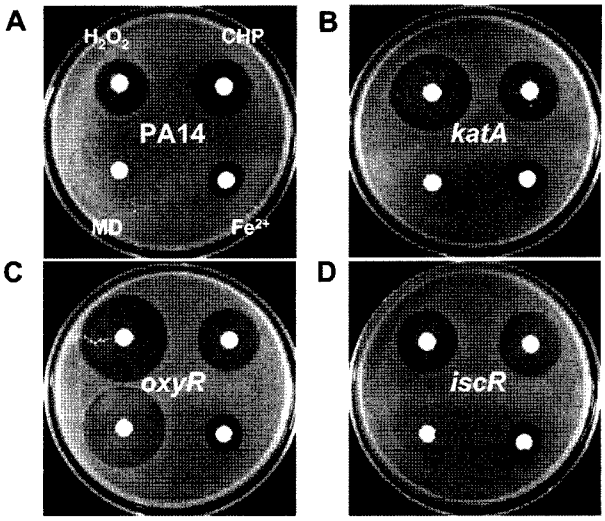

Fig. 3. Susceptibility of *katA*, *oxyR*, and *iscR* mutants.
Lawns of WT, *katA*, *oxyR*, and *iscR* cells were generated by overlaying LB agar plates with soft (0.7%) agar containing 2×10^9 CFU of designated cells. Following 1 h air drying, 3 μl droplets of 8.8 M hydrogen peroxide, 5 M cumene hydroperoxide (CHP), 2 M menadione (MD), and 1 M ferrous chloride (Fe^{2+}) were spotted on the paper discs placed at the 1, 5, 7, 11 o'clock directions of each plate. Photographs were taken after further incubation at 37°C for 24 h.

Antioxidant Enzyme Expression in the H_2O_2-sensitive Mutants

To test whether the H_2O_2-sensitivity and the susceptibilities to related stress treatments involve the reduced expression of the antioxidant enzymes such as catalases and SODs, we peformed enzyme activity staining from the logarithimic and stationary phase cultures of the H_2O_2-sensitive mutants (Fig. 4). The *oxyR* mutant displayed similar levels of KatA during the normal aerobic planktonic growth in LB broth, although it was highly sensitive to the H_2O_2 and MD treatments (Fig. 3C). It is likely that other antioxidant defence systems are attributed to the oxidant sensitivity of the *oxyR* mutant, since OxyR regulon includes many antioxidant enzymes such as KatB and AhpCF [27] and possibly some yet unknown antioxidant systems in response to oxidative challenges. We are currently trying to determine by GeneChip analysis how many genes are affected by *oxyR* mutation in response to H_2O_2 treatment.

There might be slight increase in SOD expression in *katA* and *oxyR* mutants (Fig. 4, lanes 2 *vs.* 4 and 6). This indicates some feedback regulatory mechanism governing

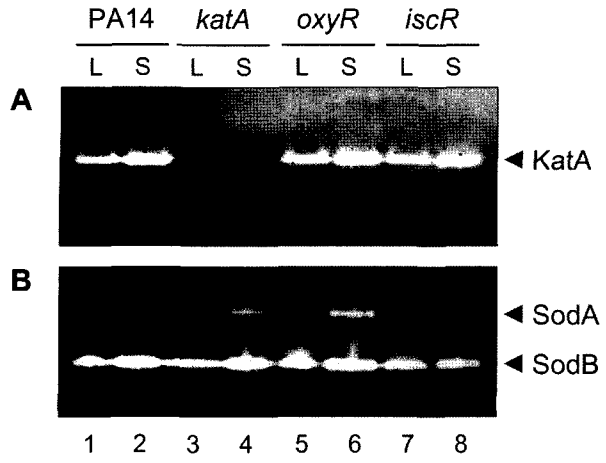

Fig. 4. Catalase/SOD expression in *oxyR* and *iscR* deletion mutants.

Detection of catalase (**A**) and SOD (**B**) activities in wild-type and mutant bacteria during the normal liquid culture. Wild-type (PA14), *katA*, *oxyR*, and *iscR* cells were grown in LB media to logarithmic growth phase (L, odd-numbered lanes) and stationary phase (S, even-numbered lanes). Cell extracts (20 μg) were electrophoresed on polyacrylamide gels, and then stained for catalase or SOD activity, as described in Materials and Methods.

SOD expression and/or activity by the accumulated H_2O_2 in those mutant bacteria, which requires further evaluation. We could not detect the KatB and KatE activities in our experimental conditions.

Most notably, the *iscR* mutant exhibited decreased KatA and SodB levels both in the logarithmic and stationary growth phases, as well as the decreased SodA level in the stationary growth phase (Fig. 4, lanes 7 and 8). The decreased level of KatA activity is likely associated with posttranslational regulation (for example, cofactor acquisition as a heme protein), since, like *E. coli* IscR, *P. aeruginosa* IscR may play a role that links oxidative stress responses and anaerobic metabolism by regulating iron-sulfur cluster assembly systems [35]. Although the *iscR* mutant was no less susceptible to MD, it is also worthwhile to demonstrate whether the dissimilar SOD level in the *iscR* mutant is associated with its decreased transcription, translation, or cofactor acquisition, considering that the SodB contains Fe as the cofactor. These genetic links between *iscR* mutation and decreased activities of antioxidant enzymes may suggest a relationship between these redox-related transcriptional regulators and the availability of free metals and the subsequent levels of the antioxidant enzyme activities requiring the metal cofactors during the oxidative stress treatments.

Acknowledgments

This work is supported by a Korea Research Foundation Grant (2005-015-C00434) to Y.-H. Cho. The authors (except for the corresponding author) were the recipients of the BK21 Fellowship from the Korean Ministry of Education and Human Resources Development.

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