Gene Expression and Physiological Role of *Pseudomonas aeruginosa* Methionine Sulfoxide Reductases during Oxidative Stress

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Pseudomonas aeruginosa PAO1 has two differentially expressed methionine sulfoxide reductase genes: *msrA* (PA5018) and *msrB* (PA2827). The *msrA* gene is expressed constitutively at a high level throughout all growth phases, whereas *msrB* expression is highly induced by oxidative stress, such as sodium hypochlorite (NaOCl) treatment. Inactivation of either *msrA* or *msrB* or both genes (*msrA msrB* mutant) rendered the mutants less resistant than the parental PAO1 strain to oxidants such as NaOCl and H₂O₂. Unexpectedly, *msr* mutants have disparate resistance patterns when exposed to paraquat, a superoxide generator. The *msrA* mutant had a higher paraquat resistance level than the *msrB* mutant, which had a lower paraquat resistance level than the PAO1 strain. The expression levels of *msrA* showed an inverse correlation with the paraquat resistance level, and this atypical paraquat resistance pattern was not observed with *msrB*. Virulence testing using a *Drosophila melanogaster* model revealed that the *msrA*, *msrB*, and, to a greater extent, *msrA msrB* double mutants had an attenuated virulence phenotype. The data indicate that *msrA* and *msrB* are essential genes for oxidative stress protection and bacterial virulence. The pattern of expression and mutant phenotypes of *P. aeruginosa msrA* and *msrB* differ from previously characterized *msr* genes from other bacteria. Thus, as highly conserved genes, the *msrA* and *msrB* have diverse expression patterns and physiological roles that depend on the environmental niche where the bacteria thrive.

Pseudomonas aeruginosa is a Gram-negative metabolically versatile bacterium commonly found in the environment, including hospitals. This bacterium can grow under nutrient-limited conditions and in stressful environments (1). In health care settings, *P. aeruginosa* is an opportunistic pathogen responsible for serious bacterial infections in immunocompromised hospitalized patients with cancer, AIDS, cystic fibrosis, and burn injuries. During infection, *P. aeruginosa* is exposed to toxic levels of reactive oxygen species (ROS) generated by host innate immune cells. In the environment outside the host, *P. aeruginosa* is exposed to ROS produced by other microbes; moreover, ROS is generated as a by-product of its own aerobic metabolism (2). The ROS can oxidize cellular macromolecules, including proteins and nucleic acids, leading to cell damage and eventual bacterial cell death. To survive oxidative stress, *P. aeruginosa* has evolved a wide variety of mechanisms to protect itself and to repair the damage caused by ROS toxicity. Several antioxidant enzymes capable of degrading ROS, such as catalases, superoxide dismutases, peroxiredoxins, alkyl hydroperoxide reductases, organic hydroperoxide resistance protein, and thiol peroxidase, have been characterized (2–6). *P. aeruginosa* also produces a number of enzymes that are required for repairing oxidative DNA damage (7). However, the repair system for oxidized proteins in this microorganism is poorly understood.

Both free and bound methionine (Met) are highly sensitive to oxidation by ROS. In biological systems, methionine oxidation often results in the formation of methionine sulfoxide (MetSO) that could alter protein conformation and/or inactivate protein functions. Methionine sulfoxide can be directly repaired by methionine sulfoxide reductase (Msr), which catalyzes a reverse reaction to convert MetSO to Met. Generally, oxidation of Met produces two S- and R-MetSO diastereoisomers that are referred to as Met-S-SO and Met-R-SO. The reduction of Met-S-SO and Met-

R-SO to Met is catalyzed by MsrA and MsrB, respectively. Both MsrA and MsrB can reduce either free or protein-bound MetSO residues (8, 9). Msr catalyzes reduction of MetSO to Met through oxidation of its reactive cysteine residue to form a sulfenic acid intermediate prior to forming an intramolecular disulfide bond between the catalytic Cys and the resolving Cys residues (8, 10). The oxidized enzymes are reactivated using the NADPH/thioredoxin reductase/thioredoxin system (10, 11). Both *msrA* and *msrB* are highly conserved and exist in a diverse range of organisms (12, 13). In pathogenic bacteria, a lack of functional *msrA* not only increases sensitivity to oxidative stress but is also associated with an attenuated virulence phenotype (14–19). Oxidation of bacterial Met to MetSO has been shown to contribute to pathogen killing in neutrophils by the myeloperoxidase (MPO) system, probably via generation of hypochlorous acid (HOCl), a potent oxidant inducing Met oxidation (20).

In this study, the roles of *P. aeruginosa msrA* (PA5018) and *msrB* (PA2827) in oxidative stress protection, gene expression profiles, and bacterial virulence were evaluated.

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TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s) ^a	Source or reference
<i>P. aeruginosa</i> strains		
PAO1	Wild-type strain	ATCC 15692
<i>msrA</i> strain	<i>msrA</i> mutant, derivative of PAO1 in which <i>msrA</i> was disrupted by pKNOCK <i>msrA</i> , Gm ^r	This study
<i>msrB</i> strain	<i>msrB</i> mutant, derivative of PAO1 in which a part of <i>msrB</i> was deleted by Cre- <i>loxP</i> system	This study
<i>msrA msrB</i> strain	<i>msrA msrB</i> mutant, derivative of <i>msrB</i> in which <i>msrA</i> was disrupted by pKNOCK <i>msrA</i> , Gm ^r	This study
<i>E. coli</i> strains		
DH5α	λ ⁻ φ80d <i>lacZ</i> Δ <i>M15</i> <i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>hsdR17</i> (r _K ⁻ m _K ⁺) <i>supE44</i> <i>relA1</i> <i>deoR</i> Δ(<i>lacZYA-argF</i>) <i>U169</i>	Stratagene Inc. (USA)
BW20767	<i>leu-63::IS10</i> <i>recA1</i> <i>creC510</i> <i>hsdR17</i> <i>endA1</i> <i>zbf-5</i> <i>uidA</i> (Δ <i>MluI</i> :: <i>pir</i> ⁺ <i>thi</i> RP4-2-tet::Mu-1kan::Tn7	60
Plasmids		
pKNOCK _{Gm}	Suicide vector, Gm ^r	24
pKNOCK <i>msrA</i>	pKNOCK _{Gm} containing a 351-bp fragment of the <i>msrA</i> coding region, Gm ^r	This study
pBBR1MCS-4	Medium-copy-number expression vector, Ap ^r	26
pBBR <i>msrA</i>	Full-length <i>msrA</i> coding region cloned into pBBR1MCS-4, Ap ^r	This study
pBBR <i>msrB</i>	Full-length <i>msrB</i> coding region cloned into pBBR1MCS-4, Ap ^r	This study
pUFR047	Low-copy-number expression vector, Ap ^r	27
pUFR <i>msrA</i>	Full-length <i>msrA</i> coding region cloned into pUFR047, Ap ^r	This study
pUFR <i>msrB</i>	Full-length <i>msrB</i> coding region cloned into pUFR047, Ap ^r	This study
pCM351	Vector containing the <i>loxP</i> -Gm ^r - <i>loxP</i> region, Gm ^r	25
pCM157	Vector containing the Cre-encoding gene, Tc ^r	25

^a Gm^r, gentamicin resistance; Ap^r, ampicillin resistance; Tc^r, tetracycline resistance.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. All bacterial strains and plasmids used in this study are listed in Table 1. Both *Escherichia coli* and *Pseudomonas aeruginosa* strains were aerobically cultivated in Luria-Bertani (LB) broth at 37°C with shaking at 180 rpm unless otherwise stated. The medium for *E. coli* growth was supplemented with 100 μg ml⁻¹ ampicillin (Ap), 15 μg ml⁻¹ gentamicin (Gm), 30 μg ml⁻¹ kanamycin (Km), or 15 μg ml⁻¹ tetracycline (Tc) as required, whereas the medium for *P. aeruginosa* was supplemented with 200 μg ml⁻¹ carbenicillin (Cb), 25 μg ml⁻¹ chloramphenicol (Cm), 30 μg ml⁻¹ Gm, or 200 μg ml⁻¹ Tc as required. To produce synchronous growth, an overnight culture was inoculated into fresh LB medium to give an optical density at 600 nm (OD₆₀₀) of 0.1. Exponential-phase cells (OD₆₀₀ of approximately 0.6 after 3 h of growth) were used in all experiments.

Molecular techniques. General molecular techniques were performed using standard protocols (21). Transformation of plasmids into *P. aeruginosa* strains was carried out using electroporation as previously described (22).

Northern blot analysis. Total RNA isolation, agarose formaldehyde gel electrophoresis, blotting, and hybridization were performed as previously described (21). For analysis of *msrA* and *msrB* expression, 10 μg and 50 μg of purified total RNA were loaded into the gel. Radioactively labeled probes were prepared using [α-³²P]dCTP and a DNA labeling bead (Amersham, GE Healthcare). A 351-bp fragment of the *msrA* coding region and a 193-bp fragment of the *msrB* coding region used as gene-specific probes were amplified from pBBR*msrA* using primers BT3002 (5'-ACAGGTGCTGTTTCGGCATGG-3') and BT3003 (5'-AGTTCGGCTGGAAGCTCTC-3') and from pBBR*msrB* using primers BT2203 (5'-ATCTATCATTCACCTGCTG-3') and BT2204 (5'-GGAAGACGTGCCCCAGGTG-3').

Quantification of *msrA* and *msrB* expression by endpoint RT-PCR and real-time PCR. Total RNA samples were prepared from PAO1 cultures induced with 1 mM H₂O₂, 1 mM paraquat (PQ), or 0.2% NaOCl for 15 min that were treated with DNase I (Fermentas, Lithuania) according to the manufacturer's protocol. First-strand cDNA synthesis was accomplished using RevertAid Moloney murine leukemia virus (M-MuLV) reverse transcriptase (Fermentas, Lithuania) with random hexamer primers and total RNA (1 μg). Endpoint reverse transcription-PCR (RT-PCR)

was performed to preliminarily determine the expression levels of *msrA* and *msrB* using primers BT3002 (5'-ACAGGTGCTGTTTCGGCATGG-3') and BT3003 (5'-AGTTCGGCTGGAAGCTCTC-3') for *msrA* and primers BT2203 (5'-ATCTATCATTCACCTGCTG-3') and BT2204 (5'-GGAAGACGTGCCCCAGGTG3') for *msrB* and 10 ng of cDNA as a template for 25 cycles, as follows: 95°C for 15 s, 55°C for 15 s, and 72°C for 15 s. The 16S rRNA gene, amplified by RT-PCR using primers BT2781 (5'-GCCCCGACAAGCGGTGGAG-3') and BT2782 (5'-ACGTATCCACACCTTCTC-3'), was used as an internal control to normalize cDNA samples. The PCR products were visualized by 1.8% agarose gel electrophoresis. Real-time PCR experiments were performed as described previously (23). Briefly, 10 ng of cDNA was used as a template in a Kapa SYBR Fast quantitative PCR (kit containing Master Mix 2×; ABI Prism) reaction in an Applied Biosystems StepOnePlus real-time PCR system. All reactions were set up according to the manufacturer's instructions. In this test, the comparative threshold cycle (*C_T*) method is used to determine the relative target quantity in the samples. With the comparative *C_T* method, the StepOne software measures the amplification of the targets, *msrA* and *msrB*, and of the endogenous control, 16S rRNA, in the samples under induced conditions and in the reference sample (the uninduced condition). Measurements are normalized using the endogenous control. The cycling parameters used were as follows: enzyme activation at 95°C for 20 s, followed by 40 cycles at 95°C for 3 s and 60°C for 60 s. All real-time PCR experiments were performed in independent triplicate trials.

Construction of *P. aeruginosa msrA* mutant. Insertional inactivation of *msrA* (PA5018) was performed using the pKNOCK suicide plasmid vector (24). The internal DNA fragment of the *msrA* coding region was PCR amplified with primers BT3002 (5'-ACAGGTGCTGTTTCGGCATGG-3') and BT3003 (5'-AGTTCGGCTGGAAGCTCTC-3'), using genomic DNA isolated from wild-type PAO1 as the template. The 351-bp PCR product was cloned into pKNOCK-Gm cut with SmaI, generating pKNOCK_{Gm}*msrA*. This recombinant plasmid was introduced into PAO1 by conjugation. The transconjugants were selected for by the gentamicin resistance (Gm^r) phenotype. A single homologous recombination event between the *msrA* fragment on pKNOCK_{Gm}*msrA* and its counterpart on the chromosome results in the insertion of the plasmid and inactivation of *msrA*. The *msrA* mutant was confirmed by PCR and Southern blot analyses.

Construction of *P. aeruginosa* *msrB* mutant. The unmarked deletion of PAO1 *msrB* was constructed using gene replacement with the Cre-*loxP* system (25). A 1,453-bp fragment covering the entire *msrB* gene and the 5' and 3' flanking regions was amplified with the primers BT1187 (5'-CCTGGATCCGCTGGTAATACTGCAT-3') and BT190 (5'-GGTGCTCATTCAAGCAGCG-3') and cloned into pUC18 digested with EcoRI-BamHI (blunted with Klenow fragment), yielding pUC*msrB*. A KpnI and SacII fragment containing a Gm^r cassette flanked with *loxP* sites from pCM351 (25) was cloned into pUC*msrB* cut with KpnI and SacII, yielding pUCΔ*msrB*::Gm. Digestion of pUC*msrB* with KpnI and SacII resulted in a deletion of 305 bp of the *msrB* coding region. The pUCΔ*msrB*::Gm was transferred into PAO1, and the transformants were selected for a double crossover event by means of the Gm^r and Cb-sensitive phenotypes. To obtain an unmarked *msrB* mutant, a Cre recombinase expression plasmid, pCM157 (25), was transferred into the transformant, and Cre-*loxP* recombination facilitated the excision of the Gm^r cassette. The pCM157 was cured by growing cells under a nonselective condition for several generations. The deletion of *msrB* was confirmed by PCR and Southern blot analyses.

Construction of a double *msrA* *msrB* mutant. The *msrA* *msrB* mutant was constructed by the transfer of pKNOC_{Gm}*msrA* into the Δ*msrB* mutant and selected by the Gm resistance phenotype. The double *msrA* *msrB* mutant was confirmed by PCR and Southern blot analyses.

Cloning of full-length *msr* genes. Full-length *msrA* and *msrB* genes were PCR amplified from PAO1 genomic DNA using the primers BT3004 (5'-CGCGAGTCATTCGCCTACC-3') and BT3005 (5'-CTCGACTGGCTGGTGGCG-3') for *msrA* and the primers BT2201 (5'-CGAGGTAGAGCCGATGAG-3') and BT2202 (5'-CGGGTCGTCGCTCTCTAGCT-3') for *msrB*. The PCR products were cloned into the medium-copy-number expression vector pBBR1MCS-4 (26) and the low-copy-number expression vector pUFR047 (27) at the SmaI site. The resulting plasmids were named pBBR*msrA*, pBBR*msrB*, pUFR*msrA*, and pUFR*msrB*.

Viability test with H₂O₂ and NaOCl exposures. Exponential-phase cultures were normalized to an OD₆₀₀ of 0.5 before being treated with 100 mM H₂O₂ or 0.3% NaOCl for 30 min at 37°C. After treatment, cells were washed twice with fresh LB broth. Cells that survived the treatment were scored using a viable cell count. The resistance levels against H₂O₂ or NaOCl were expressed as percent survival, defined as the percentage of CFU in treated cultures divided by the percentage of CFU in untreated cultures.

Determination of oxidant resistance levels. A plate sensitivity assay was performed to determine the oxidant resistance level as previously described (28). Briefly, exponential-phase cells were 10-fold serially diluted in fresh LB medium, and 10 μl of each dilution was spotted onto LB plates containing 500 μM hydrogen peroxide (H₂O₂), 1,600 μM cumene hydroperoxide (CuOOH), 300 μM *N*-ethylmaleimide (NEM), or 175 μM paraquat (PQ). The plates were incubated at 37°C for 18 h before the numbers of CFU were scored. The resistance level against an oxidant was expressed as the percent survival, defined as the percentage of CFU on the plates containing oxidant divided by the percentage of CFU on plates without oxidant. For anaerobic conditions, the culture plates were incubated in a 3.5-liter anaerobic jar containing an anaerobic gas pack (AN35; AnaeroGen, Oxoid, United Kingdom) and an anaerobic indicator and placed in a 37°C incubator for 48 h before the CFU were scored.

SOD activity assay. Xanthine-xanthine oxidase-coupled reduction of cytochrome *c* was used to measure total superoxide dismutase (SOD) activity (29). One unit of SOD activity was defined as the amount of enzyme required to inhibit the rate of reduction of cytochrome *c* by 50%.

***Drosophila* virulence tests.** The virulence of *P. aeruginosa* was investigated using the *Drosophila melanogaster* feeding assay performed as previously described (30, 31). Each of the exponential-phase cultures of each *P. aeruginosa* strain was adjusted to an OD₆₀₀ of 0.5 before 800 μl of the homogenized bacterial cells was overlaid to completely cover the surface of the preservative-free corn flour *Drosophila* medium (500 ml of water, 62 g of corn flour, 8 g of yeast extract, 40 g of sugar, and 4 g of agar) at the

bottom of a glass fly culture vial. Approximately 1-week-old adult flies were starved for 3 h prior to the feeding assay. Twenty flies each were added to every vial, and each strain of *P. aeruginosa* was tested at least for three replications. Then, all of the tested flies were incubated at 25°C for 15 h before the number of viable flies was observed. The experiments were performed in double-blind fashion and analyzed from nine experiments of three different batches of flies.

RESULTS AND DISCUSSION

Identification and transcription organization of *msr* genes in *P. aeruginosa*. The *P. aeruginosa* PAO1 genome (32) contains PA5018, a 648-bp open reading frame (ORF) annotated as *msrA* encoding the peptide methionine sulfoxide reductase A. The deduced amino acid sequence of *P. aeruginosa* MsrA shares 58.5%, 56.7%, and 56.3% identity with MsrA from *E. coli* (33), bovine (*Bos taurus*) (34), and mouse (*Mus musculus*) (35), respectively (Fig. 1A). Lower percent identities (27.7% to 34.3%) between *P. aeruginosa* MsrA and MsrA from *Erwinia pyrifoliae*, *Neisseria meningitidis*, and *Saccharomyces cerevisiae* were observed (Fig. 1A). The MsrA signature motif GCFWG (8, 34) and a thioredoxin binding domain at the C terminus (36) are conserved in *P. aeruginosa* MsrA. The *msrA* gene is located 93 bp downstream of PA5017, a hypothetical protein. Analysis of transcriptional organization of these genes by Northern blotting and RT-PCR using primers located on the PA5017 and *msrA* genes indicated that they are separately transcribed (Fig. 2A and data not shown). A 399-bp PA2827 annotated as methionine sulfoxide reductase B (*msrB*) shares 70.0% identity with MsrB from *E. coli* (Fig. 1B). The highly conserved catalytic motif RXCN of MsrB (36, 37) is also conserved in PAO1 MsrB. The *msrB* gene is located immediately upstream of a putative operon consisting of the PA2826 gene, a glutathione peroxidase homolog (*gpx*) and the PA2825 gene, a transcription regulator (*ospR*) (38). Northern blot analyses of *msrA* and *msrB* showed that these genes are transcribed mostly as a monocistronic transcript (Fig. 2A).

The expression patterns of *msrA* and *msrB* in response to oxidative stress. There is a clear need to comprehensively analyze the patterns of gene expression of both *msrA* and *msrB* during standard growth phases and in response to stresses. Currently, analyses of bacterial *msrA* and *msrB* show highly diverse patterns of gene expression in terms of growth phase and stress response. Bacteria in different growth phases confront different levels of oxidative stress resulting from physiologically generated ROS from aerobic metabolism. The *msr* genes in some bacteria have been shown to have growth phase-dependent expression (15, 33). Endpoint RT-PCR was performed to determine the levels of *msrA* and *msrB* at early log, mid-log, early stationary, and late stationary phases of growth of PAO1 cultures. The levels of *msrA* and *msrB* transcripts were not significantly altered during the different growth phases tested (data not shown).

Adaptive gene expression is an important component of bacterial defense against stresses. The expression of many genes involved in oxidative stress protection and repair processes are frequently induced by exposure to oxidants. The expression patterns of *msrA* and *msrB* in the PAO1 strain cultivated under inducing concentrations of various oxidants were determined using real-time PCR. The results illustrated that exposure of PAO1 to H₂O₂ (1 mM), paraquat (1 mM), and sodium hypochlorite (NaOCl; 0.2%) did not induce expression of *msrA* (Fig. 2C). Similarly, no significant induction of *msrB* expression was observed in para-

FIG 1 Genetic organization and multiple amino acid sequence alignments of *P. aeruginosa* MsrA and MsrB. Genetic organization and alignments of MsrA (A) and MsrB (B) from *P. aeruginosa* with other characterized enzymes from various organisms were performed using the CLUSTALW program. The arrow direction indicates the transcription orientation. Dark gray boxes indicate the MsrA or MsrB signature motif. The asterisk, colon, and period symbols indicate identical residues, conserved substitutions, and semiconserved substitutions, respectively. Values at right are percent identities of the aligned proteins with the *P. aeruginosa* protein. *gpx*, glutathione peroxidase homolog (PA2826); and *ospR*, oxidative stress response and pigment production regulator.

FIG 2 Expression analysis of *msrA* and *msrB* during normal growth and in response to oxidants. (A) Autoradiograms of Northern blots of uninduced and 0.2% NaOCl-induced samples probed with either radioactively labeled *msrA* or *msrB*. Equal amounts of purified total RNA from uninduced (UN) and 0.2% NaOCl induced (IN) samples were loaded into each lane (10 μ g for *msrA* and 50 μ g for *msrB*). The number below each band represents the fold change in band intensity relative to the level of the uninduced culture, determined using densitometric analysis. (B) Real-time RT-PCR analysis of *msrA* and *msrB* expression. Total RNA was isolated from uninduced exponential-phase cultures. Real-time RT-PCR and subsequent analysis were performed on equal amounts of cDNA (10 ng) prepared from total RNA samples as described in Materials and Methods. Data shown are fold change of expression levels using *msrB* as the reference sample. (C) Real-time RT-PCR analysis of the expression levels of *P. aeruginosa* *msrA* and *msrB* in cultures induced with either 1 mM H₂O₂, 1 mM PQ, or 0.2% NaOCl. Oxidant induction, RNA isolation, real-time RT-PCR, and subsequent analyses were performed as described in Materials and Methods. Data presented are means $\pm$ standard deviations of three independent experiments. The asterisk indicates statistically significant difference ($P < 0.01$) compared with the uninduced condition.

quat- and H₂O₂-treated samples. In contrast, NaOCl treatment of PAO1 highly induced *msrB* expression by 17.6-fold (Fig. 2C). PAO1 cultures were induced with H₂O₂ at concentrations ranging from 250 μ M to 10 mM. H₂O₂ treatment has been shown to oxidize free and bound Met, leading to the formation of both S and R epimers of MetSO. In many bacteria H₂O₂ is reported to be a potent inducer of *msr* expression (39–41). At all H₂O₂ concentrations tested, no induction of either *msrA* or *msrB* expression was observed (data not shown). Hence, lack of H₂O₂ induction of *msrA* and *msrB* expression could be due partly to the highly efficient H₂O₂ removal systems of PAO1 coupled with the uninduced levels of *msrA* and *msrB* that are most likely sufficient to repair H₂O₂ oxidation of Met in both protein and free forms.

The pattern of oxidants capable of inducing *msrB* and the oxidant concentration required for induction do not match any known oxidative stress sensor/transcriptional regulators in *P. aeruginosa*. We tested the NaOCl induction of *msrB* in mutants of transcription regulators involved in oxidative stress response, such as *oxyR*, *ohrR*, *ospR*, and *iscR*. The results showed that the fold induction by NaOCl of *msrB* expression in these mutants was induced to levels similar to the level attained in PAO1 (data not shown).

Recently, the hypochlorite-specific transcriptional regulator YjiE has been reported (42). A mutant of a *yjiE* homolog (PA5428) was constructed, and NaOCl induction of *msrB* was tested (S. Mongkolsuk, K. Saninjuk, and W. Wirathorn, unpublished data). The PA5428 mutant gave a similar level of NaOCl-induced *msrB* expression as a similarly treated wild-type PAO1, indicating that PA5428 (*yjiE* homolog) played no role in regulation of hypochlorite-induced *msrB* expression (data not shown). Moreover, sigma factor-mediated regulation of *msr* has been reported; for example,

FIG 3 Determination of H₂O₂ and NaOCl resistance levels. The resistance levels of *P. aeruginosa* strains against 100 mM H₂O₂ or 0.3% NaOCl were determined using a viability test performed as described in Materials and Methods. The percent survival was defined as the percentage of the CFU with treatment divided by the percentage of CFU without treatment. Data presented are means $\pm$ standard deviations of three independent experiments. The asterisk indicates a statistically significant difference ($P < 0.01$) compared with the PAO1 strain.

msrA and *msrAB* are members of σ^F and σ^E regulons in *Caulobacter crescentus* and *N. meningitidis*, respectively (43, 44). Further experiments are required to investigate the contribution of sigma factors in *msrA* gene regulation in *P. aeruginosa*. Thus, at present, it is not known which transcriptional regulator controls *msrB* expression in *P. aeruginosa*. In a few bacteria where oxidant-induced expression of *msr* genes has been characterized, the transcriptional regulator responsible for this adaptation has not been identified (15, 41, 45).

MsrA and MsrB recognize the S and R forms of MetSO and catalyze their conversion to Met. However, MsrA has much higher efficiency in repairing oxidation of free Met than MsrB (46). Under normal physiological growth conditions, the basal level of *msrA* transcripts was 17.2-fold higher than the level of *msrB* transcripts (Fig. 2B). This indicates that MsrA is present at relatively high concentrations throughout various growth phases and during exposure to oxidants, ensuring that it is ready to repair both free and bound MetSO formation upon oxidant exposure. In contrast, *msrB* expression is maintained at a low level under normal physiological conditions. Once the bacteria are exposed to oxidants such as NaOCl from external sources, rapid induction (over 17.6-fold) of *msrB* expression occurs (Fig. 2C). NaOCl is a strong oxidant, and in aqueous solutions, NaOCl ionizes to generate Na⁺ and hypochlorite (OCl⁻), which rapidly forms hypochlorous acid (HOCl). Hence, NaOCl and derivatives could directly react with various macromolecules. Moreover, as a secondary effect, NaOCl treatment also generates arrays of ROS (47, 48).

Mutations in *msrA* and *msrB* altered resistance levels to NaOCl and H₂O₂. HOCl is an important component of the host-generated immune response against bacterial infection. Thus, during their interactions with the host, bacteria are exposed to harmful levels of highly reactive HOCl. Recently, genes involved in protection from oxidative stress have been implicated in the role of protecting bacteria from NaOCl toxicity (39, 49, 50). In this study, experiments were performed to elucidate the role of *P. aeruginosa* *msr* genes in protecting the bacteria from NaOCl toxicity. Specifically, resistance levels to NaOCl of PAO1 and various *msr* mutants were assessed. The results showed that *msrA*, *msrB*, and *msrA msrB* mutants were 10-, 10²-, and 10³-fold more sensi-

tive to 0.3% NaOCl treatment, respectively, than the PAO1 parental strain. The difference in the reduction of NaOCl resistance levels in the single *msrA* and *msrB* mutants and the additive effects of reduction in NaOCl resistance levels in the double mutant indicate that *msrA* and *msrB* have functionally different capacities in protection against NaOCl toxicity. Next, the ability of *msrA* or *msrB* in a medium-copy-number expression vector (pBBR1MCS-4) to complement the reduced NaOCl resistance phenotype in PAO1 and the mutant strains was determined. First, PAO1 strains harboring either pBBR*msrA* or pBBR*msrB* have similar resistance levels to NaOCl as PAO1 harboring a vector control. This result indicates that high expression levels of either *msrA* or *msrB* do not confer additional resistance to NaOCl. Second, pBBR*msrA* and pBBR*msrB* plasmids fully complemented the NaOCl-sensitive phenotypes of *msrA* and *msrB* mutants to resistance levels attained by the parental strain, supporting the idea that the observed phenotype of the mutants was due to a lack of either MsrA or MsrB. Because MsrA and MsrB have different substrate ranges and biochemical properties, the experiments were extended to test cross-complementation using either pBBR*msrA* or pBBR*msrB* in the single mutants. The results showed that pBBR*msrB* partially complemented the NaOCl-sensitive phenotype of the *msrA* mutant to a level that was 5-fold lower than that of PAO1, while pBBR*msrA* could also partially complement the phenotype of the *msrB* mutant to a level 10-fold lower than that of the wild-type strain (Fig. 3). In addition, the ectopic expression of either pBBR*msrA* or pBBR*msrB* also partially complemented the NaOCl resistance levels of the double *msrA msrB* mutant to a resistance level 10-fold lower than that of PAO1 (Fig. 3).

Exposure of bacteria to NaOCl results in the generation of many types of ROS (47, 48, 51), which could oxidize both free and bound Met residues in proteins, leading to a loss of biological functions. One could assume that, upon Met oxidation, S and R epimers of MetSO are formed at equal efficiencies, and the repair capacity would reside in the quantity and efficiency of MsrA and MsrB. The expression of either *msrA* or *msrB* from an expression vector did not confer additional resistance to NaOCl upon wild-type PAO1 strains, supporting the notion that NaOCl treatment produced other forms of cellular damage in addition to oxidation of Met. The mutational analysis revealed that both *msrA* and *msrB*

FIG 4 Determination of paraquat resistance levels. The resistance levels of *P. aeruginosa* strains against 0.175 mM paraquat were determined using a plate sensitivity assay as described in Materials and Methods and are expressed as percent survival. The percent survival was defined as the percentage of CFU on plates containing paraquat divided by the percentage of CFU on plates without paraquat. Data presented are means $\pm$ standard deviations of three independent experiments. The asterisk indicates a statistically significant difference ($P < 0.01$) compared with the PAO1 harboring pBBR1MCS-4 or the pUFR047 vector control.

have differential capacities to reduce free or peptidyl MetSO in the repair of oxidized methionine resulting from the NaOCl treatment. The ability of ectopic expression of *msrA* and *msrB* to partially cross-complement the NaOCl-sensitive phenotypes of the *msrA*, *msrB*, and double *msrA msrB* mutants supports the separate roles the two enzymes have in the overall protection of Met from oxidants. The partial cross-complementation phenotype could arise from the ability of each enzyme to functionally substitute for the lack of either MsrA or MsrB activities or the interconversion between S and R epimers of MetSO.

P. aeruginosa is likely to encounter toxic levels of H_2O_2 during interaction with hosts and in the environment. H_2O_2 can oxidize both free and bound Met (52). Genetic analyses in other bacteria have shown that inactivation of either *msrA* or *msrB* renders the mutants less resistant to H_2O_2 treatment (45, 53). The resistance levels to H_2O_2 in PAO1 and various *msr* mutants were determined. Unexpectedly, the single mutants *msrA* and *msrB* did not show significant alteration in H_2O_2 (100 mM) resistance levels under the conditions tested. Nonetheless, the H_2O_2 resistance level of the *msrA msrB* double mutant was 8-fold more sensitive to H_2O_2 treatment than PAO1. This small but reproducible reduction in the H_2O_2 resistance level of the double mutant could be fully complemented by either pBBR*msrA* or pBBR*msrB*. The lack of H_2O_2 phenotypes in single *msr* mutants and a small increase in H_2O_2 sensitivity in the *msrA msrB* mutant are both likely due to the presence of numerous peroxide-scavenging enzymes, such as catalases, peroxiredoxins, and alkyl hydroperoxide reductases, which efficiently removed H_2O_2 to mitigate the peroxide oxidation of Met. A minor susceptibility to H_2O_2 could be observed only when methionine sulfoxide reductases were totally deficient. The data support a minor role for *msrA* and *msrB* in protecting the cell from H_2O_2 -induced death. This is consistent with the strategy used by many bacteria in that oxidant removal has a primary role over the repair of oxidatively damaged macromolecules. Once the oxidant removal capacity is exceeded and oxidation of macromolecules begins, repair of the oxidatively damaged molecules becomes a major component of the oxidant removal systems that protect the bacteria.

No significant alteration in resistance levels was observed in the *msrA*, *msrB*, and *msrA msrB* mutants when the strains were treated with 1.6 mM CuOOH and 300 μ M NEM.

Inactivation of either *msrA* or *msrB* disparately alters the mutants' ability to cope with superoxide stress.

Superoxide anion-mediated oxidation of Met residues in proteins and free Met has been reported (53, 54). In other bacteria, inactivation of *msr* genes has been shown to increase sensitivity to the superoxide generator paraquat (53); therefore, the physiological roles of *P. aeruginosa* *msrA* and *msrB* in superoxide stress protection were evaluated. The resistance levels of *P. aeruginosa* strains against paraquat were determined. Unexpectedly, the *msrA* mutant was 70-fold more resistant to paraquat (175 μ M) killing than the PAO1 parental strain (Fig. 4). In contrast, an *msrB* mutant was 10^2 -fold more sensitive to paraquat than a parental strain (Fig. 4). The resistance level of the double *msrA msrB* mutant was equivalent to the sum of the resistance levels of the *msrA* and *msrB* mutants and comparable to the level of PAO1. These findings support a notion that the altered paraquat phenotypes of *msrA* and *msrB* mutants arise from disparate pathways. A recent observation indicated that paraquat toxicity to bacteria results either directly from the redox cycling property of the drug or from superoxide anions generated as by-products of the reaction (55). We extended the investigation to determine the probable mode of paraquat killing of various *msr* mutants by performing a paraquat plate sensitivity assay under aerobic and anaerobic conditions using LB medium supplemented with 1% nitrate. The PAO1, *msrA*, *msrB*, and double mutant strains had similar patterns of paraquat resistance under aerobic growth conditions, performed using either LB or LB medium plus nitrate. In contrast, under anaerobic growth conditions, the addition of 175 μ M paraquat to LB medium plus nitrate did not significantly reduce the percent survival of either wild-type PAO1 or *msr* mutants (Fig. 5). Moreover, when a higher paraquat concentration (500 μ M) was used in the anaerobic plate sensitivity assay, no reduction in the survival of PAO1 and mutant strains was detected (Fig. 5). Clearly, paraquat-induced killing of *P. aeruginosa* required aerobic conditions; hence, the toxicity likely arises from superoxide anions generated by the drug. Paraquat resistance levels have been known to correlate with the ability of bacterial cells to remove superoxide anions and superoxide dismutase (SOD) activity. Total SOD activity was measured in the *msrA*, *msrB*, and double mutants, all grown aerobically. The results showed nonsignificant differences in the levels of total SOD activity in the wild-type and mutant strains tested (data

FIG 5 Determination of paraquat resistance levels under aerobic and anaerobic conditions. A plate sensitivity assay was performed using LB medium with and without supplementation of 1% KNO₃ and incubated under aerobic and anaerobic conditions. The concentrations of paraquat were 0.175 mM for aerobic experiments and 0.5 mM for anaerobic experiments. The resistance levels of *P. aeruginosa* strains against paraquat were determined using a plate sensitivity assay and are expressed as percent survival as defined in the legend of Fig. 4. Data presented are means $\pm$ standard deviations of three independent experiments. The asterisk indicates a statistically significant difference ($P < 0.01$) compared with PAO1.

not shown). Therefore, alterations in levels of paraquat resistance of the *msrA* and *msrB* mutants are independent of total SOD enzyme activity.

We extended the investigation by examining the ability of *msrA* and *msrB* to complement and cross-complement the paraquat resistance phenotypes of the *msrA* and *msrB* mutants. Paraquat resistance levels of PAO1, *msrA*, *msrB*, and the double *msrA msrB* mutant strains harboring either pBBR*msrA*, pBBR*msrB*, or a vector control were measured. Unexpectedly, high levels of expression of *msrA* from pBBR*msrA* reduced the percentage of survival after paraquat treatment by 10³-fold for the PAO1 and *msrA* strains and by more than 10-fold for the *msrB* and *msrA msrB* mutants relative to strains harboring the vector control (Fig. 4). We examined the effect of lowering *msrA* expression levels using pUFR047 (a low-copy-number expression vector; 2 to 4 copies) instead of pBBR1MCS (a medium-copy-number vector; 30 to 40 copies) (56). Decreasing the copy number of the expression vectors, and hence reducing *msrA* expression levels, in strains harboring pUFR*msrA* resulted in a 10²-fold increase in percent survival of PAO1 and the *msrA* mutant. However, in the *msrB* strain and the double *msrA msrB* mutant, there was a 50-fold increase in the percent survival with paraquat treatment compared to the strains harboring a medium-copy-number vector (Fig. 4). The results from expression analysis of pBBR*msrA* and pUFR*msrA* clearly reveal a correlation between the levels of *msrA* expression and the magnitude of reduction in the paraquat resistance levels in all strains tested, regardless of the host genetic background. The effects of *msrB* expression in PAO1 and the three *msr* mutant strains were also examined. The expression of *msrB* in the *msrA* mutant lowered its paraquat resistance level by 8-fold, whereas both pBBR*msrB* and pUFR*msrB* could fully complement the paraquat-sensitive phenotype of the *msrB* mutant to levels similar to the level of PAO1. There were no alterations in the paraquat resistance phenotype associated with *msrB* expression levels in PAO1 and all mutant strains harboring either pBBR*msrB* or pUFR*msrB*.

The phenotypes of paraquat resistance of the *msrA* mutant and strains with overexpression of *msrA* have not been observed in

other bacteria and are highly unexpected. Theoretically, high expression of a gene encoding a repair enzyme for oxidized Met such as MsrA should enhance the resistance level against oxidant treatments. In contrast, the results here showed that the level of MsrA correlated with a reduction in the paraquat resistance level, as illustrated by two independent observations. First, inactivation of *msrA* resulted in an increase in the paraquat resistance level; second, a high expression level of *msrA* from the expression vectors decreased the paraquat resistance level. These observations suggest that MsrA possesses other enzymatic activity besides the methionine sulfoxide reductase function that repairs free and bound MetSO. A recent report that MsrA is also a stereospecific methionine oxidase, producing Met-S-SO, raises the possibility that *P. aeruginosa* MsrA also has additional oxidase-like activity (57). Similar to findings of a previous report (57), the depletion of thioredoxin by paraquat treatment could lead to enhanced methionine oxidase activity and the formation of MetSO in target proteins that render the bacteria more susceptible to paraquat killing.

***msrA* and *msrB* mutants are attenuated for virulence in the *Drosophila* host model.** The role of *msr* genes in virulence and pathogenicity varied widely in different bacteria. Nonetheless, *msr* genes have important roles in virulence and pathogenesis in many bacteria. Hence, the virulence of the PAO1, *msrA*, *msrB*, and double *msrA msrB* mutant strains was tested using *Drosophila melanogaster* as a host model. As shown in Fig. 6, feeding the flies with PAO1 cultures resulted in 25.0% $\pm$ 5.0% fly survival, compared with 95.0% $\pm$ 2.6% fly survival when LB medium was fed as a negative control. When either an *msrA* or *msrB* mutant was used to feed *D. melanogaster*, the fly survival increased to 64.5% $\pm$ 6.1% or 61.1% $\pm$ 8.0%, respectively. Moreover, the percent survival of the fly was further increased to 71.7% $\pm$ 4.1% when the double *msrA msrB* mutant was used in the feeding experiment. The significant increase in the percent survival of *Drosophila* flies ranging from 40 to 50% after feeding with the *msrA*, *msrB*, and the *msrA msrB* double mutant strains compared to levels with PAO1 indicated that these mutant strains have an attenuated virulence phenotype in the test model ($P < 0.01$ by Student *t* test). This pheno-

FIG 6 Virulence assay of *P. aeruginosa* strains. The virulence of *P. aeruginosa* strains was determined using the *Drosophila melanogaster* feeding method (30, 31). The flies that survived were counted after 15 h of incubation, and results are expressed as the percent viability. The percent viability was defined as the percentage of the surviving flies after 15 h of incubation with *P. aeruginosa* cell cultures. Data presented are means $\pm$ standard deviations of three independent experiments. The asterisk indicates a statistically significant difference ($P < 0.01$) compared with PAO1.

type in both the *msrA* and *msrB* mutants could be rescued in the complemented strains expressing functional a copy of each gene (Fig. 6). The attenuated virulence phenotype of the double *msrA msrB* mutant could only be partially complemented by expression of either *msrA* or *msrB* from expression vectors (Fig. 6). These data suggest that *msrA* and *msrB* are virulence determinants for *P. aeruginosa* in the *D. melanogaster* model, and they indicate the involvement of MsrA and MsrB in *P. aeruginosa* virulence.

The roles of methionine sulfoxide reductases in virulence have been observed in other pathogenic bacteria; for example, *Salmonella enterica* serovar Typhimurium MsrA, but not MsrB, has an important role in oxidative stress protection and in virulence (58). MsrA has been described as a virulence determinant in various plant- and human-pathogenic bacteria such as *Erwinia chrysanthemi* (16), *Staphylococcus aureus* (17), *Mycobacterium smegmatis* (18), and *Mycoplasma genitalium* (19). Recently, *msrB* has been shown to have a role in virulence in *Enterococcus faecalis* (59).

Loss of either *msrA* or *msrB* in *P. aeruginosa* attenuated its ability to cause death of the flies. The greater attenuated virulence of the *msrA msrB* mutant confirmed that the two genes functioned in different pathways in protecting the bacteria from host defense responses (Fig. 6). We speculated that the phenotypic difference between the *msrA* and *msrB* mutants could be due to these genes likely having roles in different virulence pathways during the bacterial infection, which could arise from different expression patterns of the two *msr* genes and their different enzymatic activities.

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