Molecular Insights into the Impact of Oxidative Stress on the Quorum-Sensing Regulator Protein LasR*

Prapti Kafle[1], Amanda N. Amoh[1,2], Jocelyn M. Reaves[2], Emma G. Suneby, Kathryn A. Tutunjian, Reed L. Tyson, and Tanya L. Schneider[3]

From the Department of Chemistry, Connecticut College, New London, Connecticut 06320

The LasR regulator protein functions at the top of the *Pseudomonas aeruginosa* quorum-sensing hierarchy and is implicated in promoting bacterial virulence. Of note is recent evidence that this transcription factor may also respond to oxidative stress. Here, all cysteines in LasR were inspected to deduce their redox sensitivity and to probe the connection between stress response and LasR activity using purified LasR and individual LasR domains. Cys[79] in the ligand binding domain of LasR appears to be important for ligand recognition and folding of this domain to potentiate DNA binding but does not seem to be sensitive to oxidative stress when bound to its native ligand. Two cysteines in the DNA binding domain of LasR do form a disulfide bond when treated with hydrogen peroxide, and formation of this Cys[201]-Cys[203] disulfide bond appears to disrupt the DNA binding activity of the transcription factor. Mutagenesis of either of these cysteines leads to expression of a protein that no longer binds DNA. A cell-based reporter assay linking LasR function with β-galactosidase activity gave results consistent with those obtained with purified LasR. This work provides a possible mechanism for oxidative stress response by LasR and indicates that multiple cysteines within the protein may prove to be useful targets for disabling its activity.

The Gram-negative bacterium *Pseudomonas aeruginosa* is an opportunistic human pathogen that establishes chronic infections in immunocompromised patients, with persistent pulmonary colonization in individuals with cystic fibrosis (1–3). Like many bacteria, *P. aeruginosa* employs quorum-sensing (QS)[4] machinery to communicate local population density through the exchange of self-produced signaling molecules (4). QS contributes to the development of acute infections by coordinating the production of multiple virulence factors, including elastase and pyocyanin, and the formation of biofilms (5–7). QS in *P. aeruginosa* is largely mediated by *N*-acyl L-homoserine lactone autoinducer molecules, biosynthesized by LuxI-type synthases, and detected by LuxR-type regulator proteins (8). The two major systems responsive to acylhomoserine lactones (AHLs) in *P. aeruginosa* are LasI/LasR and RhlI/RhlR. LasI and RhlI are LuxI-type synthases, whereas LasR and RhlR are LuxR-type regulatory proteins. These AHL-dependent systems are also closely connected to signaling governed by 2-alkyl-4-quinolones in *P. aeruginosa* (9).

The regulator protein LasR, with its cognate synthase LasI, functions at the top of the *P. aeruginosa* quorum-sensing hierarchy (10). Like other LuxR-type proteins, this transcription factor is composed of two domains (11). The amino-terminal ligand binding domain (LBD) specifically recognizes its autoinducer, *N*-(3-oxo-dodecanoyl)-L-homoserine lactone (3O-C$_{12}$-HSL), promoting protein folding and dimerization, whereas the DNA binding domain (DBD) recognizes target sites to activate downstream gene expression. There has been significant interest in understanding LasR, as its inhibition represents a promising antivirulence strategy for the treatment of *P. aeruginosa*. One prevalent mode of inhibition is the development of non-native small molecules shown to disrupt LasR function through the reduction of virulence factors produced (12–14). A number of these analogs appear to inhibit LasR through competition with the native 3O-C$_{12}$-HSL because they are often structurally similar.

Recent work has also revealed a somewhat unexpected role for LasR in oxidative sensing (15). A proteome-wide screen of *P. aeruginosa* to detect H_2O_2-sensitive cysteines identified Cys[79] of LasR. Subsequent experiments in *P. aeruginosa* indicated that a LasR-C79A mutant strain showed decreased motility and proteolysis, highlighting Cys[79] as important for LasR QS function. The link between LasR and oxidative stress response was supported by Northern hybridization analysis that revealed that the RNA level of *lasI*, under LasR control, was reduced after cells were treated with H_2O_2. Complementation studies with a LasR deletion strain showed that LasR function was restored when complemented with wild-type plasmid *p-lasR* but diminished by about half when cells were treated with 5 mM H_2O_2. Interestingly, complementation with mutant plasmid *p-lasR-C79S* was only partially successful but did not change significantly in the presence of oxidant. Complementation with *p-lasR-C79A* resulted in negligible LasR activity even in the absence of oxidant. Taken together, these findings suggest that LasR responds to both its autoinducer and oxidative stress.

Numerous bacterial proteins have been shown to respond to oxidative stress through redox-sensitive thiols, including the OxyR, HypT, and MgrA transcription factors (16–18). Although these proteins are important for regulating pathogen

[4] The abbreviations used are: QS, quorum-sensing; AHL, acylhomoserine lactone; LBD, ligand binding domain; 3O-C$_{12}$-HSL, *N*-(3-oxo-dodecanoyl)-L-homoserine lactone; DBD, DNA binding domain; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); Tm, melting temperature; OP1, Operator 1.

response to potentially damaging oxidants, they are not known to be associated with QS. In addition to the recent finding that LasR may be governed by both oxidants and QS autoinducers, several other bacterial regulators have been linked to a similar dual function. *Staphylococcus aureus* AgrA has been shown to form an intramolecular disulfide bond within its DBD when oxidized, leading to a reduction in DNA binding affinity (19). Additionally, *Escherichia coli* SdiA, a LuxR homolog like LasR, forms an intermolecular disulfide bond within its DBD, covalently linking two monomers and altering promoter binding affinity when oxidized with the addition of 2 mM H_2O_2 in EMSAs (20). These studies provide additional evidence for a possible intersection between QS and oxidative sensing in bacterial regulator proteins.

In this work, we probed the mechanism for the reported LasR sensitivity to oxidative stress to further characterize this important transcriptional regulator at the biochemical level. Our exploration of Cys[79] revealed that this residue is important for ligand recognition and protein folding, but it does not seem to be sensitive to oxidative stress when LasR is autoinducer-bound and folded. Instead, we have identified two cysteines in the DBD of LasR, Cys[201] and Cys[203], which appear to modulate DNA binding affinity through the formation of an intramolecular disulfide bond in the presence of H_2O_2. Additionally, mutagenesis of either of these residues leads to poor protein overexpression and loss of DNA binding, underscoring their critical role in functional LasR. Results from these *in vitro* assays with purified protein were further supported by parallel studies using *E. coli* transformed with a plasmid containing both the *lasR* gene and a *lasB-lacZ* detection system (21). Experiments with this reporter gene system also demonstrated the critical importance of Cys[201] and Cys[203] for LasR function in a cell-based assay and indicated the down-regulation of LasR activity in the presence of H_2O_2. Taken together, our results add to the growing collective understanding of LasR, supporting a possible additional role for this protein as an oxidative sensor and suggesting new ways in which this pathogenic regulator may be handicapped.

Experimental Procedures

Cloning, Expression, and Purification of LasR, LBD, and DBD—The *P. aeruginosa* PA01 *lasR* gene was amplified by PCR from a *lasR*-containing pDONR201 vector obtained from the Harvard Medical School PlasmID Repository. Using incorporated NotI and NcoI restriction sites, the *lasR* insert was ligated into the pET28b (Novagen) expression vector to yield plasR. Similarly, the *lasR* fragment (nucleotides 1–519) corresponding to the LasR LBD (amino acid residues 1–173) (22) was amplified and cloned into the NdeI/NotI sites of the pET24b vector to yield pLBD. The *lasR* fragment (nucleotides 523–720) corresponding to amino acid residues 175–240 with added *N*-terminal Met was amplified and cloned into the NotI/NcoI sites of pET28b to yield pDBD. Generation of Cys[79], Cys[188], Cys[201], and Cys[203] mutants was achieved using the QuikChange Lightning site-directed mutagenesis kit (Agilent Technologies). All constructs were sequenced to confirm correct gene insertion and desired mutations (DNA Analysis Facility, Yale University).

For expression of all proteins, BL21(DE3) cells were transformed with the expression plasmid of interest and grown in Luria broth medium supplemented with 30 μg/ml kanamycin. For the expression of LasR and LBD, growth medium was additionally supplemented with 20 μM 3O-C_{12}-HSL (Sigma-Aldrich) or non-native AHLs. Cultures were grown to $A_{600} = 0.8$ at 37 °C, protein expression was induced by the addition of isopropyl 1-thio-β-D-galactopyranoside to 400 μM, and growth continued for an additional 24 h at 23 °C. Cells were harvested by centrifugation, with pellets stored at −80 °C.

Harvested cells resulting from 0.5 liter of culture were resuspended in 10 ml of lysis buffer (25 mM Tris (pH 7.5), 200 mM NaCl, and 10% (v/v) glycerol). Lysis buffer was supplemented with 1 mM tris(2-carboxyethyl)phosphine for the purification of LasR and DBD. Resuspended cells were lysed by sonication and clarified by centrifugation, and the resulting supernatant was incubated with 0.25 ml of nickel-nitrilotriacetic acid-agarose resin (Qiagen) for 2 h at 4 °C. The resin was decanted into a column and washed with 5 ml of 5 mM imidazole in lysis buffer. C-terminally His_6-tagged LasR, LBD, or DBD protein was eluted with a step gradient of 30, 60, 100, 250, and 500 mM imidazole in lysis buffer. Fractions containing the desired protein as judged by SDS-PAGE were pooled and dialyzed against dialysis buffer (50 mM sodium phosphate (pH 7.0), 200 mM NaCl, and 10% (v/v) glycerol, with 1 mM DTT added for LasR and DBD purification). LasR purified without DTT in dialysis buffer was not an active DNA-binding protein.

Synthesis of Non-native AHLs—AHL derivatives were prepared by coupling acyl carboxylic acids with L-homoserine lactone in the presence of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide as described previously (23). Product identity and purity were confirmed by ^{1}H NMR (500 MHz Agilent DD2-500 NMR), with comparison to known characterization data for each compound (24).

SDS-PAGE Analysis of LBD—To measure the impact of oxidants on LBD dimerization, 25 μM LBD or LBD C79S was combined with 2.5 mM H_2O_2, 2.5 mM cumene hydroperoxide, or water as a control and incubated for 3 h at 25 °C. LBD and LBD C79S samples were diluted in non-reducing SDS sample buffer and separated using 12% polyacrylamide gels (Bio-Rad). Samples containing 100 mM DTT in sample buffer were incubated for 10 min at 25 °C prior to electrophoresis. Detection, excision, and elution of protein bands for mass spectrometric confirmation of molecular weight followed established methods (25). LBD expressed in the presence of non-native AHLs was also diluted in non-reducing SDS sample buffer and resolved on a 12% polyacrylamide gel.

DTNB Assay—LBD (25 μM) was combined with 0–200 μM H_2O_2 in native (100 mM sodium phosphate and 1 mM EDTA (pH 8.0)) or denaturing (6 M guanidine hydrochloride, 100 mM sodium phosphate, and 1 mM EDTA (pH 8.0)) buffer and incubated for 1 h at 25 °C. To detect free thiols, 180 μM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) was added and incubated for 15 min at 25 °C, and the absorbance of each sample was measured at 412 nm. The concentration of 2-nitro-5-thiobenzoate, released upon reaction between free thiols and DTNB, was calculated using this absorbance and known extinction coeffi-

cients for 2-nitro-5-thiobenzoate under native and denaturing conditions (26).

Circular Dichroism Spectroscopy—Far-UV CD spectra of wild-type and mutant LBD (15 μM in 100 mM sodium phosphate (pH 7.0)) expressed in the presence of varied AHLs were measured with a Jasco J-810 spectropolarimeter using a 0.1-cm path length quartz cuvette. Data were acquired at 10 °C, and spectra represent the average of four scans for each sample. Temperature-dependent CD analysis at 222 nm from 10 – 90 °C at a rate of 1 °C/min permitted determination of melting temperature (Tm) using the Jasco Spectra Analysis software to fit data.

Electrophoretic Mobility Shift Assays—Binding reactions were performed in binding buffer (0.7 mM KH_2PO_4, 2.15 mM Na_2HPO_4, 1.35 mM KCl, 20 mM NaCl, 0.5 mM EDTA, 0.05% (v/v) Triton X-100, 5% (v/v) glycerol, and 10 μg/ml salmon sperm DNA (pH 7.4)). A 43-mer DNA duplex containing the *lasB* OP1 site (5′-ATCAAGGCTACCTGCCAGTTCTGGCA-GGTTTGGCCGCGGGTTC-3′ annealed to its complementary strand) with 5′ modification of the top strand with fluorescein (6-FAM, Integrated DNA Technologies) was used for all binding assays. For quantitative binding assays, serial dilutions of LasR, LasR mutants, and DBD were incubated with 100 nM *lasB* OP1 for 30 min at 25 °C before binding reactions were applied to a nondenaturing 6% polyacrylamide DNA retardation gel (Invitrogen) with 0.5× Tris borate-EDTA as the electrophoresis running buffer. The amounts of complexed and free DNA were visualized and quantified using a Gel Doc EZ system (Bio-Rad). Equilibrium dissociation constants were determined as described previously (27). Assays to test the impact of H_2O_2 on DNA binding were performed under the same conditions, with incubation with oxidant for 30 min at 25 °C either before or after incubation with target DNA as noted. The low millimolar concentration of H_2O_2 used in these EMSA assays is common practice for the *in vitro* evaluation of the impact of oxidant on regulator protein-DNA binding affinity (18 – 20, 28 – 30) and is required in part to overcome the 1 mM DTT necessary for the purification and storage of active LasR. To evaluate recovery of binding with DTT, samples were incubated for an additional 30 min at 25 °C in the presence of excess reducing agent (25 mM DTT). DBD (64 μM) was reacted with 45 mM iodoacetamide for 1 h at 25 °C to give alkylated DBD for gel shift analysis.

Mass Spectrometric Characterization of LasR—All mass spectral analyses were performed by tandem HPLC-electrospray ionization mass spectrometry (LC-MS) using a Thermo Finnigan Surveyor HPLC system and a Thermo Scientific LTQ XL mass spectrometer. In preparation for LC-MS analysis, 2.5 μM LasR C79S/C188S and LasR C79S each were incubated with 3 mM H_2O_2 for 2 h at 25 °C, followed by reaction with 45 mM iodoacetamide for 1 h at 25 °C to cap any remaining free thiols. Digestion of LasR C79S/C188S proceeded with the addition of Glu-C (Promega) to 3.5 ng/μl in 50 mM NH_4HCO_3 (pH 8.0) with incubation at 37 °C for 18 h. Digested proteins were subjected to LC-MS, with ions of interest selected and subjected to collision-induced dissociation (MS/MS) to confirm peptide identity.
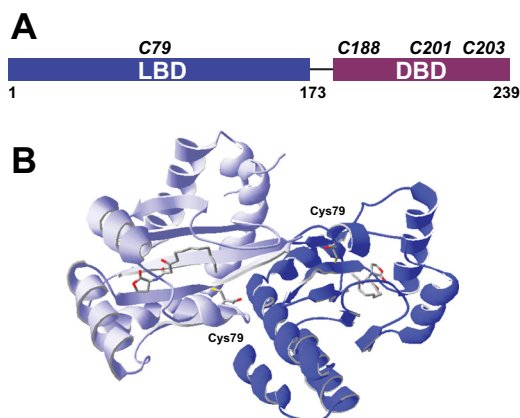

FIGURE 1. **Cysteine composition of LasR.** *A*, domain structure of LasR with the relative positions of all LasR cysteines shown. *B*, dimeric crystal structure (22) of the LBD with $3O-C_{12}$-HSL bound by each monomer and both Cys[79] residues shown. Within each monomer, the terminal carbon of $3O-C_{12}$-HSL is proximal to Cys[79] (4.3 Å), whereas the two cysteines in the dimeric complex are 21.6 Å apart.

β-Galactosidase Reporter Assays—XL10-Gold *E. coli* cells (Agilent Technologies) were transformed with plasmid pKDT17 to yield a reporter strain coupling the *lasR* gene with a *lasB::lacZ* translational fusion (21). pKDT17 was a gift from Peter Greenberg (Addgene plasmid 27503). Site-directed mutagenesis of pKDT17 to convert LasR Cys[79], Cys[188], Cys[201], and Cys[203] to serine was achieved using the QuikChange Lightning site-directed mutagenesis kit (Agilent Technologies) and confirmed by DNA sequencing (DNA Analysis Facility, Yale University).

Using this reporter strain, LasR activity was quantified as a function of β-galactosidase activity as measured in Miller assays (31) using the modified procedure reported previously (32). Starting with a 1/1000-fold dilution of an overnight culture, the reporter strain was grown at 37 °C with shaking in LB medium supplemented with ampicillin (200 μg/ml) to $A_{600} = 0.8 – 1.2$. Cultures also included 20 μM $3O-C_{12}$-HSL and 10 – 100 μM H_2O_2 as noted. Subsequent dilution, permeabilization, and β-galactosidase activity assays were performed as described previously (32), with measured enzyme activity similarly converted to Miller units to account for any variance in culture density or reaction times.

Results

Impact of Oxidative Stress on the LasR Ligand Binding Domain—Previous work identified Cys[79] of LasR, the sole cysteine within the LasR ligand binding domain (Fig. 1), as sensitive to oxidative stress in a proteome-wide screen of *P. aeruginosa* (15). This report provided evidence that LasR activity is impacted by oxidative stress, particularly in cell-based assays, but did not delineate the underlying protein chemistry of this redox response. We were interested in probing this biochemical mechanism further and learning the specifics of how LasR function may span both quorum-sensing and redox-sensing pathways. To take a close look at Cys[79], we expressed and purified the LasR LBD (amino acids 1–173) in the presence of its native autoinducer, $3O-C_{12}$-HSL, yielding soluble protein. Although there are numerous possible outcomes for the reaction of a thiol such as Cys[79] with an oxidizing agent, one conceivable oxidative stress response mechanism is the formation
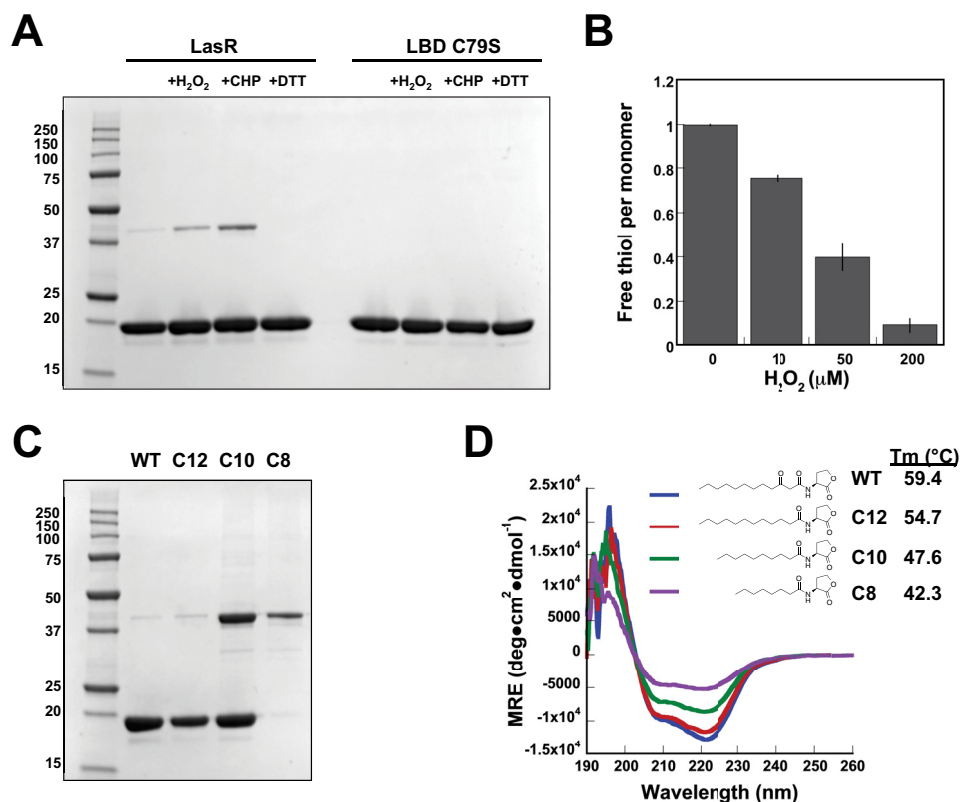
FIGURE 2. **Impact of oxidative stress and unnatural ligands on LasR LBD.** *A*, SDS-PAGE of 25 μM LBD under non-reducing conditions showing both monomer and dimer bands. Protein bands were excised from the *third lane* of a duplicate gel and subjected to LC-MS to confirm the predicted molecular weight of monomer (20,453.0 Da observed and 20,451.1 Da calculated) and dimer (40,906.5 Da observed and 40,900.2 Da calculated) LBD. Dimer formation is enhanced in the presence of 2.5 mM H_2O_2 and cumene hydroperoxide (*CHP*), whereas only monomer LBD is observed in the presence of 100 mM DTT or with the LBD C79S mutant. *B*, DTNB quantification of free thiols under denaturing conditions illustrates the impact of 10–200 μM H_2O_2 on 25 μM LBD. *Error bars* denote standard deviation for triplicate data. *C*, non-reducing SDS-PAGE of LBD expressed in the presence of 3O-C_{12}-HSL (*WT*) and the non-native ligands C_{12}-HSL, C_{10}-HSL, and C_8-HSL. *D*, wavelength-dependent CD spectra comparing relative folding of 15 μM LBD expressed in the presence of varied AHLs. The thermal stability (Tm) of each was also determined.

of a disulfide bond between two reactive thiols, here the linking of two Cys[79] residues. Interestingly, we saw evidence of some dimeric LBD when the protein was purified under non-reducing conditions and evaluated on a denaturing, non-reducing SDS-PAGE gel (Fig. 2*A*). This result suggests a disulfide bond between monomers. Dimer formation was promoted in the presence of oxidants such as H_2O_2 and cumene hydroperoxide but reversed in the presence of the reducing agent DTT. Additionally, mutagenesis of Cys[79] to serine (LBD C79S) confirmed that Cys[79] was required for formation of the observed covalent dimer. These results led us to probe this dimer further to determine its importance for oxidative sensing by LasR. Because the two Cys[79] in the known dimeric, autoinducer-bound LBD crystal structures (22, 33) are ~21 Å apart (Fig. 1*B*), a significant conformational change or unfolding of the protein would be necessary to permit the formation of a disulfide bond. Such a structural change, though, could impact the ability of LasR to activate transcription.

To further characterize the reactivity of the Cys[79] thiol, we completed a DTNB assay for free thiols under native and denaturing conditions with 25 μM LBD. No labeling was observed for native LBD, but denaturation of the protein led to stoichiometric labeling of one thiol per monomer as anticipated. Addition of H_2O_2 from 10–200 μM readily protected the denatured LBD from labeling by DTNB (Fig. 2*B*), promoting the formation

of dimeric LBD as detected by LC-MS (40,899.8 Da observed and 40,900.2 Da calculated). Similar experiments with H_2O_2 were performed with native LBD, revealing neither the formation of dimeric LBD nor any other change in mass, suggesting that the thiol is largely protected from oxidation when the LBD is folded and ligand-bound. Additionally, no alkylation was observed by LC-MS when the native LBD was treated with excess iodoacetamide. The proximity of Cys[79] to the terminal carbon of 3O-C_{12}-HSL (22) (Fig. 1*B*) suggests that this thiol might be important for ligand recognition, and others have reported covalent modification of Cys[79] when LBD is expressed in the presence of electrophilic probes derived from the native AHL structure, placing reactive functional groups proximal to the thiol (34, 35). Cys[79] appears to be most sensitive to oxidative stress when not bound to its native ligand, and perhaps the dimer observed by SDS-PAGE (Fig. 2*A*) reflects the reaction of two LBD monomers denatured by SDS gel sample buffer.

Although we and others (22) have found that it is not possible to purify soluble LBD expressed in the absence of ligand, we moved toward an approximation of ligand-free LBD by incrementally shortening the acyl chain length of ligands added to LBD expression cultures. We successfully expressed and purified the LBD in the presence of a series of AHLs differing in acyl chain length and presence of the 3-oxo group. Gel analysis (Fig. 2*C*) of these samples under non-reducing conditions revealed a
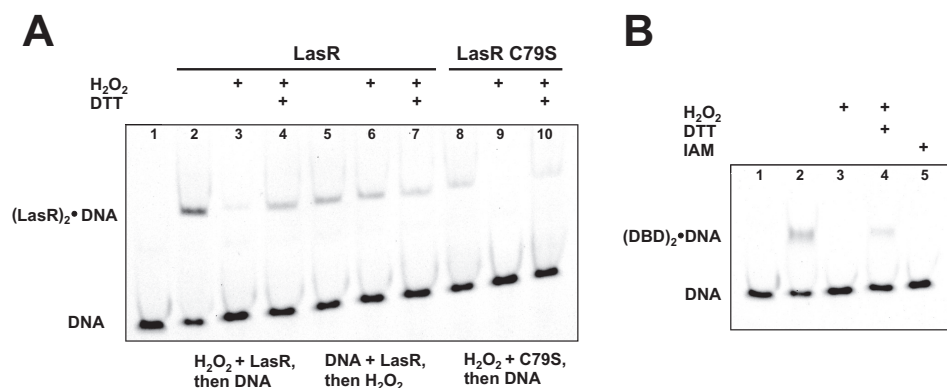
FIGURE 3. **Oxidative stress impacts LasR-DNA binding.** *A*, representative EMSA with 0.5 μM LasR and 5.0 μM LasR C79S. Target *lasB* OP1 is shown alone (*lane 1*). Both proteins shifted DNA in the absence of oxidant (*lanes 2, 5,* and *8*), and the addition of 7.5 mM H_2O_2 significantly reduced DNA binding observed (*lanes 3* and *9*). The concentration of H_2O_2 used in these assays is similar to that reported in prior EMSA studies to evaluate the impact of oxidant on transcription factor-DNA binding (18–20, 28–30). The incubation of LasR with DNA for 30 min prior to the addition of oxidant appears to protect LasR from oxidation, preserving DNA binding (compare *lanes 3* and *6*). DNA binding for both proteins is restored with the addition of 25 mM DTT following incubation with H_2O_2 (compare *lanes 3* and *4* for LasR and *lanes 9* and *10* for LasR C79S). *B*, representative EMSA with 14 μM LasR DBD indicates binding of this domain to *lasB* OP1 (*lane 2*) that is disrupted in the presence of 7.5 mM H_2O_2 (*lane 3*) but restored with the addition of 25 mM DTT (*lane 4*). DBD reacted with iodoacetamide (*IAM*) prior to the binding assay did not show DNA binding (*lane 5*).

trend toward dimer formation as AHL acyl chains shortened, suggesting a role for the native 3O-C_{12}-HSL in mediating native LBD folding and perhaps blocking disulfide bond formation. The yield of soluble protein also decreased with shorter AHLs, and no soluble protein was produced with C_6-HSL or C_4-HSL. CD results also indicate that the secondary structure and thermal stability of LasR LBD are impacted by changes to acyl chain length (Fig. 2*D*). Our results join previous findings suggesting that autoinducer acyl side chain length is particularly important in dictating the stimulation of LasR activity (36) and recent work cataloging hydrogen-bonding interactions in the LasR LBD critical for LasR activation and inhibition (37, 38).

We also investigated the impact of Cys[79] mutagenesis on LBD structure by measuring the thermal stability of LBD mutants C79S (Tm = 55.1 °C) and C79A (Tm = 55.7 °C), finding that replacement of Cys[79] does lower thermal stability for these mutants expressed in the presence of native 3O-C_{12}-HSL compared with wild-type LBD (Tm = 59.4 °C). Expression and purification of C79A LBD in the presence of non-native C_{10}-HSL gave still lower thermal stability (Tm = 43.8 °C), a finding that parallels the diminished stability observed for wild-type LBD in the presence of shortened ligands (Fig. 2*D*). Taken together, our experiments with the LasR LBD suggest that Cys[79] is important for protein folding and ligand recognition, and may form a disulfide-linked dimer in the presence of oxidative stress. Additional experiments with full-length LasR were required to probe how Cys[79] oxidation might be linked to the function of LasR as a transcriptional activator.

Oxidative Stress Impacts LasR-DNA Binding Affinity—The full-length LasR protein with a C-terminal His$_6$ tag was expressed and purified in the presence of 3O-C_{12}-HSL, yielding soluble protein. EMSA was used to measure the interaction between LasR and one of its known target sequences, the *lasB* operator 1 (OP1) site (39). Addition of H_2O_2 to binding reactions reduced LasR-DNA binding, particularly when oxidant was added to LasR prior to the addition of DNA (Fig. 3*A*). Binding was recovered when excess DTT was added to the reaction, indicating that the impact of H_2O_2 was thiol-specific and

reversible. These results suggest that one possible mechanism for the impact of oxidative stress on LasR as a transcriptional activator is through reduction in DNA binding affinity. Our next aim was to catalogue the biochemical transformations explaining this observed change in binding affinity.

The relevance of Cys[79] in connecting oxidative stress to LasR-DNA binding affinity was measured using mutant LasR C79S in similar EMSA experiments. The addition of H_2O_2 also reduced DNA binding affinity for this mutant (Fig. 3*A*), suggesting that Cys[79] was not completely responsible for the observed reduction in binding affinity. This somewhat unexpected finding led us to investigate the three additional cysteines found outside the ligand binding domain of LasR, as detailed below.

Interestingly, mutagenesis of Cys[79] does have a significant impact on DNA binding affinity based on results from quantitative EMSA. Replacement of Cys[79] with serine increased the apparent equilibrium dissociation constant (K_d) from 0.400 $\pm$ 0.086 μM to 48.4 $\pm$ 8.0 μM (Fig. 4). The K_d for C79A LasR also increased to 4.89 $\pm$ 0.84 μM. Although Cys[79] may not represent the thiol that is mediating oxidative stress response in native LasR, it does appear to be important for DNA binding, likely by promoting correct protein folding and dimerization in response to native autoinducer binding.

DNA Binding Domain Cys[201] and Cys[203] Respond to Oxidative Stress—Based on the EMSA experiments reported above, it seemed likely that one or more of the three cysteines within the LasR DNA binding domain (Fig. 1*A*) might be linked to the observed change in DNA-binding affinity in the presence of oxidant. We generated a construct representing only the LasR DBD (residues 175–240) to explore this portion of the protein more closely. Expression and purification of this domain with C-terminal His$_6$ tag yielded a soluble DNA-binding protein. Although this domain alone was sufficient to bind DNA, the K_d (23.8 $\pm$ 3.6 μM) was again increased over that observed for full-length LasR (Fig. 4).

Addition of H_2O_2 to DNA binding reactions containing LasR DBD yielded similar results to that seen with full-length LasR
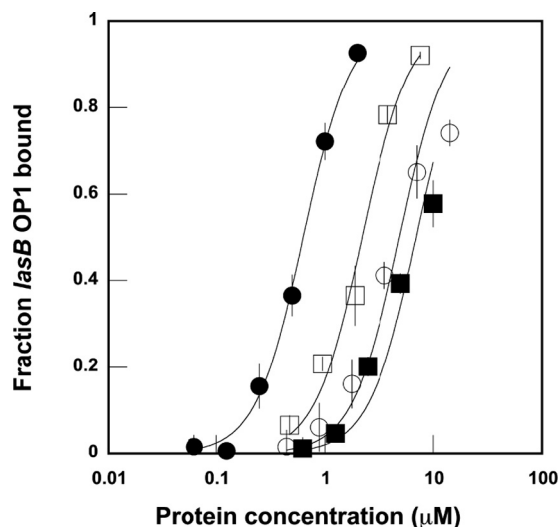
FIGURE 4. **Quantitative EMSA comparing equilibrium binding affinity for wild-type LasR (●), LasR C79S (■), LasR C79A (□), and LasR DBD (○).** The apparent K_d for each was determined by fitting data to the Langmuir equation as described in Ref. 27. *Error bars* denote standard deviation for triplicate data.

(Fig. 3*B*), confirming that one or more of the cysteines within this domain mediates LasR oxidative response. Reacting the DBD with iodoacetamide prior to EMSA experiments resulted in a triply alkylated protein (8874.4 Da observed, 8873.14 Da calculated) that no longer bound DNA (Fig. 3*B*). These results indicate that cysteines in the DBD are important for DNA binding affinity and that chemical modification of these thiols, either through oxidation or alkylation, has a decided effect on DNA binding.

To inspect each of the three DBD cysteines in turn, each was mutated to alanine in full-length LasR. C188A yielded a protein that weakly bound DNA at 6 μM, forming complexes that appeared to dissociate during electrophoresis. C201A and C203A expression resulted in proteins that gave no discernible DNA binding at 3 μM compared with completely saturated wild-type LasR binding observed at ~1 μM (Fig. 4). Generation of C201S and C203S mutants also gave proteins with no detectable DNA binding even at 7.6 μM. The mutation of Cys[201] or Cys[203] consistently resulted in poor protein overexpression yields as well, at most one-third of the yield observed for wild-type LasR and Cys[79] or Cys[188] mutants (typically 3 mg/liter). Based on these experiments that highlight the importance of proximal Cys[201] and Cys[203], we hypothesized that a disulfide bond between these two residues might represent a redox switch impacting the DNA binding affinity of wild-type LasR.

Cys[201] and Cys[203], seemingly important for LasR oxidative sensing, were probed further through a series of mass spectrometry experiments designed to assess the possibility of disulfide bond formation. LasR C79S/C188S, retaining only Cys[201] and Cys[203], was treated with H_2O_2, followed by excess iodoacetamide to cap any free thiols. LC-MS analysis revealed only the mass anticipated for LasR C79S/C188S with one disulfide bond (27,788.8 Da observed and 27,788.6 Da calculated). As a control experiment, treatment of LasR C79S/C188S with water followed by iodoacetamide yielded the mass expected for alkylation of the two free thiols (27,906.2 Da observed and 27,904.6

Da calculated). Similar experiments with LasR C79S yielded results that also supported the formation of a Cys[201]-Cys[203] disulfide bond. Here, treatment of the protein with H_2O_2 followed by iodoacetamide gave a mass equal to that predicted for one alkylated cysteine and one disulfide bond (27,860.8 Da observed and 27,861.7 Da calculated), whereas a water control experiment in this case gave a mass indicative of alkylation of all three thiols (27,980.4 Da observed and 27,977.7 Da calculated). Taken together, these results suggest that Cys[201] and Cys[203] form a disulfide bond under these oxidative conditions, whereas Cys[188] is not similarly oxidized, remaining reactive toward iodoacetamide. To further characterize the possible Cys[201]-Cys[203] disulfide bond, LasR C79S/C188S that had been treated with H_2O_2 and iodoacetamide was digested with Glu-C protease. The expected mass for the disulfide-linked fragment containing Cys[201] and Cys[203] was discerned ($(M+H)^+$ observed 965.40, calculated 965.42). LC-MS/MS fragmentation analysis confirmed the identity of this peptide fragment (Fig. 5).

LasR Reporter Assay Results Parallel in Vitro Experiments—We expanded our *in vitro* characterization of LasR by performing parallel experiments using an *E. coli*-based reporter assay. The known plasmid pKDT17 (21) includes the *P. aeruginosa lasR* gene and a *lasB::lacZ* fusion, allowing monitoring of LasR activity as reflected by its binding to the *lasB* promoter, activating production of an elastase-β-galactosidase fusion protein with resultant enzyme activity detected in a Miller assay. Here, LasR and its target *lasB* promoter are the same as components of our EMSA experiments, but this cell-based assay allowed us to interrogate our findings in a different type of system. *E. coli* (pKDT17) was cultured under a variety of conditions, testing the impact of 3O-C$_{12}$-HSL and H_2O_2 on *lasB* induction mediated by LasR. Inclusion of AHL ligand was important for detection of robust β-galactosidase activity as anticipated, and the addition of 100 μM H_2O_2 decreased observed β-galactosidase activity (Fig. 6*A*). These results are consistent with our findings that H_2O_2 impacts the DNA binding affinity of LasR. Mutagenesis of pKDT17 to convert each LasR cysteine to serine allowed inspection of the importance of each for LasR activity in this assay (Fig. 6*B*). The activity of all of the mutants was reduced compared with wild-type LasR, but C201S and C203S both showed especially low activity, similar to the wild-type construct assayed in the absence of AHL ligand. Again, these results are comparable with our findings with purified LasR mutants, particularly highlighting the essential nature of Cys[201] and Cys[203] for functional LasR. Because of the fact that Cys[201] and Cys[203] mutagenesis resulted in LasR with negligible activity under normal growth conditions, further studies to test the impact of oxidative stress on these mutants were not likely to be illustrative because their level of activity is already so low.

Modeling LasR DBD Structure—Although the structure of the LasR DBD is not known because of reported difficulties with full-length LasR protein solubility (22), we generated and evaluated a homology model of this domain using the Phyre2 protein fold recognition server (40) to consider the placement of this disulfide bond. The model, shown in Fig. 7*A*, illustrates the bond between Cys[201] and Cys[203] and predicts its position between the two helices of the helix-turn-helix motif. These
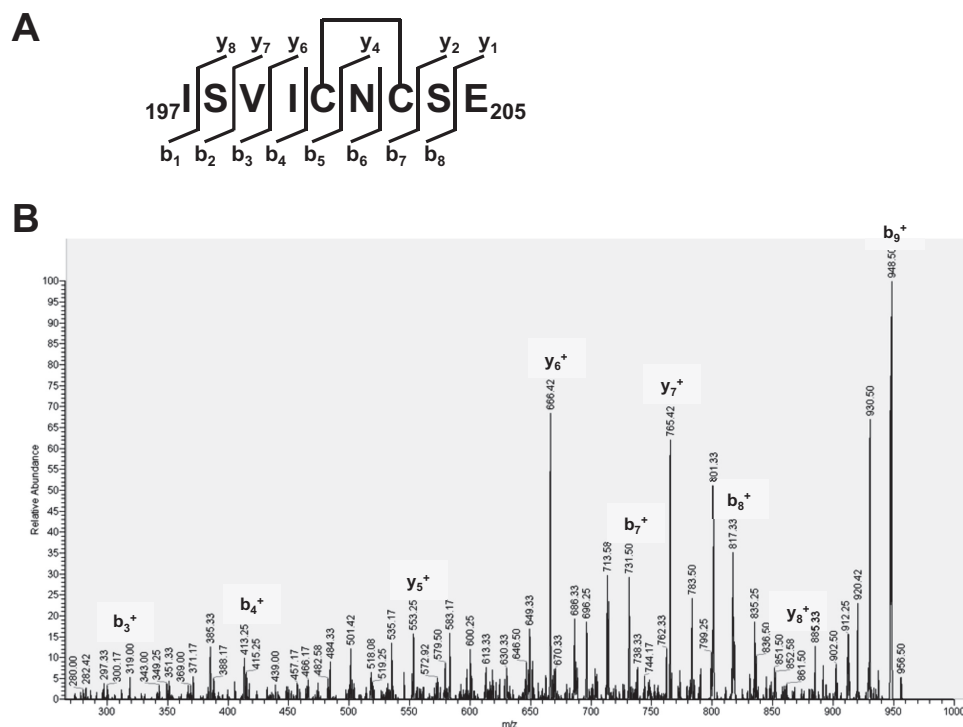
A

B

FIGURE 5. **Mass spectrometric mapping of the Cys^{201}-Cys^{203} disulfide bond in LasR.** *A*, graphical fragment map correlating the relevant peptide sequence with the observed fragmentation ions. *B*, representative MS/MS fragmentation of the ion at *m/z* 965.40 corresponding to the disulfide-linked Glu-C peptide fragment 197–205.

A

B
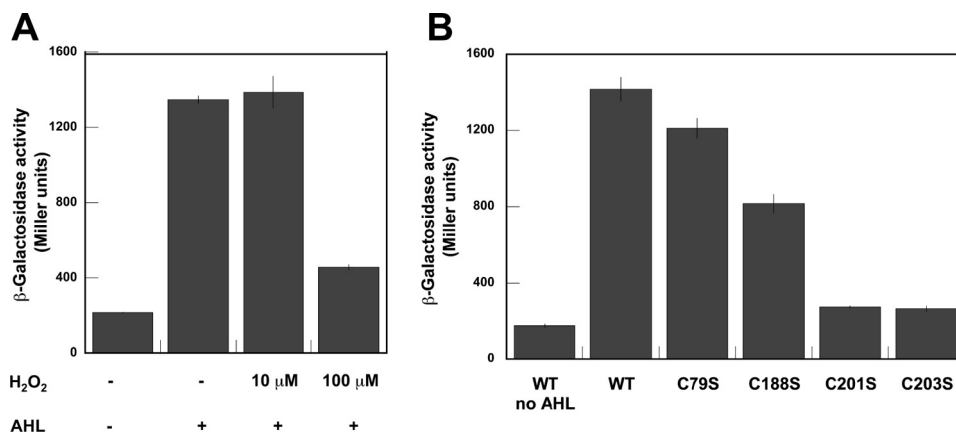
FIGURE 6. **Miller assay to detect the activity of β-galactosidase produced by the E. coli lasR lasB::lacZ reporter gene system.** *A*, impact of adding H_2O_2 and 20 μM AHL (3O-C$_{12}$-HSL) to cell cultures. *Error bars* denote standard deviation for triplicate data. *B*, impact of the indicated *lasR* gene mutations reflected in the reporter assay. Cultures were grown in the presence of 20 μM 3O-C$_{12}$-HSL except where noted. *Error bars* denote standard deviation for triplicate data.

helices correlate to "scaffold" helix α8 and "recognition" helix α9 in sequence alignment with TraR, a LuxR-type regulator from *Agrobacterium tumefaciens* whose dimeric crystal structure bound to both autoinducer and target DNA has been solved (41). To roughly approximate the position of Cys^{201} and Cys^{203} when the DBD associates with DNA, we superimposed the LasR DBD with one of the helix-turn-helix motifs in the TraR crystal structure (Fig. 7*B*). It is conceivable that the relative orientation of these helices could be impacted by the formation of a Cys^{201}-Cys^{203} disulfide bond, altering DNA binding affinity. It is also likely that one or both of these cysteines is/are important for DNA recognition or proper protein folding because conversion of either to alanine or serine yields a protein that is poorly expressed and no longer binds DNA, and alkyla-

tion of these thiols also disrupts DNA binding. Combining LasR and target DNA prior to the addition of H_2O_2 reduces the impact of the oxidant, also supporting the possibility that one or both of these cysteines interact(s) closely with DNA. We anticipate that future structural studies will permit further understanding of the roles of Cys^{201} and Cys^{203} in target site recognition.

Discussion

The QS regulator LasR is of interest because of its role in controlling *P. aeruginosa* response to increased population density, linking autoinducer concentration to up-regulation of processes that lead to heightened bacterial virulence. Inhibition of LasR, therefore, represents an attractive antivirulence strat-

FIGURE 7. **Modeling the LasR DBD.** *A*, homology model of DBD generated using the Phyre2 web server (40). The three DBD cysteines are shown, including the Cys[201]-Cys[203] disulfide bond. *B*, overlay 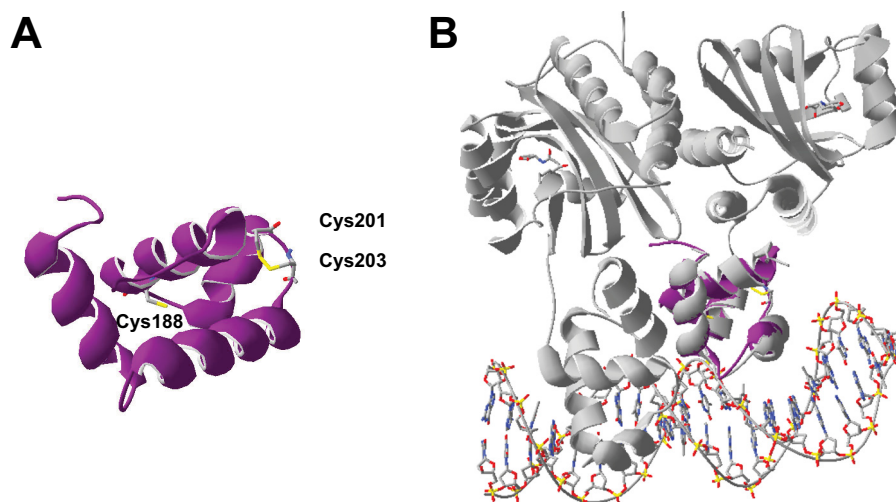of the DBD homology model (*purple*) with the TraR DBD (right monomer, shown in *gray*) within the crystal structure (41) of TraR bound to its autoinducer and target DNA.

egy, and additional understanding of LasR function and activity should enable more effective inhibition. The possibility that LasR responds to oxidative stress in addition to its known function as a QS regulator is intriguing, and we have worked to characterize this possible additional LasR function at the molecular level. Our work expands on earlier studies that demonstrated a link between oxidative stress and LasR QS efficacy in *P. aeruginosa* (15). Here we developed *in vitro* assays with purified LasR and individual LasR domains to enable us to probe the biochemical mechanism that may permit LasR to act as a redox sensor.

Systematic inspection of the four cysteines within LasR finds that Cys[79] is important for protein folding and DNA binding but does not suggest a likely mechanism for this residue to act as an oxidative sensor in native, ligand-bound LasR. Although native LBD with 3O-C$_{12}$-HSL bound does not react with DTNB or H$_2$O$_2$, denatured LBD does form a disulfide-linked dimer when exposed to oxidative stress, indicating that Cys[79] is a reactive thiol that appears to be inaccessible when native autoinducer is bound. Experiments with non-native AHLs with shorter acyl chains further demonstrate that the absence of native autoinducer may permit LasR to adopt a conformation that more readily forms a disulfide dimer. Cys[79] is conserved in some LasR homologs that similarly recognize 3O-C$_{12}$-HSL (Fig. 8), underscoring our finding that this residue aids in specific ligand recognition. We note that mutating Cys[79] significantly disrupts the enhancement in binding affinity offered by the properly folded, dimeric LasR ligand binding domain because the observed DNA binding affinity for the DBD alone only differs by a factor of two from that measured for LasR C79S, 2 orders of magnitude greater than that for wild-type LasR. It is interesting that the measured DNA binding affinity is so similar for LasR completely lacking the ligand binding domain (DBD alone) and LasR with a single ligand binding domain mutation (C79S), and these findings highlight the importance of Cys[79]. Mutagenesis of Cys[79] to serine or alanine does not abolish sensitivity of LasR to oxidative stress, though, because DNA binding affinity is still reduced for these mutant proteins under oxidizing conditions.

Although our results do not suggest that LasR Cys[79] is accessible or redox-sensitive in folded LasR, others have noted the possibility that conserved LBD cysteines might mediate the oxidative stress response in other LuxR-type regulator proteins. Recent efforts to catalogue solo *luxR* genes indicate that many of the LuxR proteins identified feature multiple conserved cysteines, particular in their LBDs, and it has been postulated that these cysteines, including Cys[79] in solo LuxR proteins, are positioned well for disulfide bond formation and possible redox regulation (42). Based on prior reports (15) and our studies, it is certainly conceivable that LasR Cys[79] has an important role *in vivo* as a reactive thiol, particularly in poorly folded LasR, and continued work will enable a full understanding of this residue.

Our experiments with the LasR DBD indicate that oxidative stress does impact the DNA binding affinity of this domain under *in vitro* reaction conditions similar to those used by others in characterizing oxidation-sensitive DNA binding proteins (18–20, 28–30). To further explore this result, we employed a cell-based reporter assay that also indicated a reduction in LasR activity in the presence of H$_2$O$_2$. These findings intersect with prior work describing the influence of oxidative stress on LasR activity in *P. aeruginosa* assays (15). Although this previous report focused on Cys[79], their results indicating a reduction in wild-type LasR activity in the presence of H$_2$O$_2$ did not delineate which of the four LasR cysteines is critical for this response and did not consider any of the DBD cysteines. Based on our studies, we now propose that the formation of a disulfide bond between Cys[201] and Cys[203] might regulate that stress response. Because mutagenesis of Cys[201] or Cys[203] yields inactive LasR in both EMSA and cell-based reporter assays, it is difficult to completely characterize their response to oxidative stress or investigate a direct link between disulfide bond formation and reduced quorum sensing in *P. aeruginosa* because complementation studies with these mutants in a LasR deletion strain are

Oxidative Stress and Quorum Sensing Regulator LasR

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|P25084|LasR    1   MALVDG--------------------FLELERSSGKLEWS-AILQKMASDLGFSKILFGLL---PKDSQDYENAFIVGNYPAAWREHYD
|P12746|LuxR    1   MKNINA-------DDTY------RI---INKIKACRSNNDIN-QCLSDMTKMVHCEYYLLAII---YPHSMVKSDISILDNYEKKWRQYYD
|B1VD86|BraR    1   MSPIL--------------------AASDETQWF-DAVAGLAGSWGFSQVLLAIL---PRPGMRLEDAYVRSNYASSWRQAYT
|Q3Y8H6|PpuR    1   MTLLVM-------DE------------LRRLSEMRFEDWL-AHLKILTRKLGYSNFLIGLK---PAPTDANQQVLIYSDYPDAWRTRYD
|Q9AH79|MupR    1   ML----------------------EDILMCETLEAWS-KVLFALAKEYGFSSVLFGVK---PTISTPFSSSTIISNYSRSWRSIYD
|A5WYC9|TraR    1   MQHW--------------------LDKLTDLAAIEGDECILKTGLADIAEHYGFTGYAY-------L-HIQHRITAVTNYHREWQSTYF
|P07026|SdiA    1   MQDKD--FFSWR--RT---------M---LLRFQRMETAEEVY-HEIELQAQQLEYDYYSLCVR---HPVPFTRPKVAFYTNYPEAWVSYYQ
|B6E4Z5|RhlR    1   MRNDGGFLLWW--DG---------L---RSEMQPIHDSQGVF-AVLEKEVRRLGFDYYAYGVR---HTIPFTRPKTEVHGTYPKAWLERYQ
|D3W065|CviR    1   MVISKPIN-ARPLPAGLTASQQWTLLEWIHMAGHIETENEL-KAFLDQVLSQAPSERLLLALGRLNNQNQIQRLERVLNVSYPSDWLDQYM

|P25084|LasR   70   ARVDPTVSHCTQSVLPIFWEP--SIY---QTRKQHEFFEEASAAGLVYGLTMPLHGARGELC-ALSLSVEAENR-AEANRFMESVLPTLWM
|P12746|LuxR   76   IKYDPIVDYSNSNHSPINWNIFENNA---VNKKSPNVIKEAKTSGLITGFSFPIHTANNGFC-MLSFAHSEKDNYIDSLF--LHACMNIPL
|B1VD86|BraR   64   AYIDPTVSHCTKHSSPLVWSP--DLF---ATTPQKSMYEEAAAHGLAGQTLPIHGPRQEAC-MLCFVNDSNPN-DKFWQHIDTALPNLVL
|Q3Y8H6|PpuR   72   AAVDPVVQHCLNANRPLLWDR--DNY---RRPSESEFFEEAAAHGLQQGLALPLHGPRGEAC-MLCLKPFESGPRATATM--IESLPMATL
|Q9AH79|MupR   65   YEVDPVVFHCLNSSLPLVWTK--ENF---INKPEQQLYEAASASGVCSGLCMPIHGPRGEFC-MLNFITDEKSP-ADVIR--SKGLPQFAL
|A5WYC9|TraR   67   VALDPVVKRARSRKHIFTWSG--EQERPSLSRDERSFYARAADFGIRSGITIPIRTANGSMS-MFTLASDKPVI--DLDR--EIDAVAAAS
|P07026|SdiA   77   LAIDPVLNPENFSQGHLMWND--DLF---S--EAQPLWEAARAHGLRRGVTQYLMLPNRALC-FLSFSRCSARE-IPILS--DELQLKMQL
|B6E4Z5|RhlR   78   GAVDPAILNGLRSSEMVVWSD--SLF---D--QSRMLWNEARDWGLCVGATLPIRAPNNLLS-VLSVARDQQNI-SSFER--EEIRLRLRC
|D3W065|CviR   94   AQHDPILRIHL-GQGPVMWEE--RFN-RAKGAEEKRFIAEATQNGMGSGITFSAASERNNICSILSIAGREPGR--NAA-----LVAMLNC

|P25084|LasR  158   ALQSGAGLA-F--EHPVSKPVVLTSREKEVLQWCAIGKTSWEISVICNCSEANVNFHMGNIRRKFGVTSRRVAAIMAVNLGLIT-L-----
|P12746|LuxR  165   LVDNYRKIN-I---ANNKSNNDLTKREKECLAWACEGKSSWDISKILGCSERTVTFHLTNAQMKLNTTNRCQSISKAILTGAIDCPYFK-N
|B1VD86|BraR  152   VIDTSQRHL-S--AHAQTLIPKLTPRERECLKVTALGKSTWEISHILNCSEAVVNFHMKNIRAKFGVNSRAAAVIATQLGIIDPG-----
|Q3Y8H6|PpuR  159   AMEGMLK---A--RIECSTPVHLTSREKEVLQWSAAGKTTWEISMILSCTTSAIDFHFKNIRRKFQVSSRQMAVLKAIQQKSITP------
|Q9AH79|MupR  151   LIESFQKIQ-SAAEKEVEKPPKLTARELECLKVVAAGKTSWEISRILSCAEVTVNFYHVTNFMRKFNAESRQHAVISGIRSGLVTP------
|A5WYC9|TraR  155   IHARISYL-RT--TPTAEDAAWLDPKEASYLRVIAVGMTMEEIADVEGVKYNSVRVKLREAMKRFDVHSKAHMIALAIRRKLI-------
|P07026|SdiA  161   SLMALMRLN-D--EIVMTPEMNFSKREKEILRWTAEGKTSAEIAMILSISENTVNFHQKNMQKKINAPNKTQVACYAAATGLI--------
|B6E4Z5|RhlR  162   LTQKLTDLE-H--PMLMSNPVCLSHRERREILQWTADGKSSGEIAIILSISESTVNFHHKNIQKKFDAPNKTLAATYAAALGLI--------
|D3W065|CviR  178   LHQAAIRVANL--PPASPSNMPLSQREYDIFHWMSRGKTNWEIATILDISERTVKFHVANVIRKLNANNRTHAIVLGMHLAMPPST-VANE
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FIGURE 8. Multiple sequence alignment of LasR and its homologs. Protein sequences shown include *P. aeruginosa* LasR (UniProt P25084), *Vibrio fischeri* LuxR (UniProt P12746), *Burkholderia kururiensis* BraR (UniProt B1VD86), *Pseudomonas putida* PpuR (UniProt Q348H6), *Pseudomonas fluorescens* MupR (UniProt Q9AH79), *A. tumefaciens* TraR (UniProt A5WYC9), *E. coli* SdiA (UniProt P07026), *P. aeruginosa* RhlR (UniProt B6E4Z5), and *Chromobacterium violaceum* CviR (UniProt D3W065). The positions of the four LasR cysteines are indicated (*stars*). Cys[79] is shared by BraR (47), PpuR (48), and MupR (49), all believed to recognize 3O-C_{12}-HSL. This is not the autoinducer for the other proteins shown. With LuxR, these proteins also share Cys[203]. Interestingly, there is no sequence homology to Cys[201] even in proteins that share Cys[203], suggesting that the potential for a disulfide bond at that position is unique to LasR among known regulator sequences. It is possible that Cys[201] in its reduced state may be an important hydrophobic amino acid, based on the conserved leucine at this position in most homologous sequences and the known hydrophobic nature of free cysteines (50).

likely to show deficient LasR-dependent quorum sensing with or without oxidative stress.

There is additional evidence that Cys[203] is important for LasR function. Although we find that both Cys[201] and Cys[203] are essential for LasR activity, Cys[203] appears to be more highly conserved based on comparison with homologous sequences (Fig. 8) and is even shared by LuxR. Furthermore, one LasR mutation observed in two different studies of clinical *P. aeruginosa* isolates is C203R, correlated with loss of LasR function as measured by elastase activity (3, 6). These clinical findings further support an important role for Cys[203]. It is not surprising that the formation of a disulfide dimer directly involving an essential DNA contact cysteine could have a significant impact on DNA binding affinity. Similar findings with transcription factor p53 have recently been reported, as Cys[275], critical for p53-DNA binding affinity, is believed to form a disulfide bond with Cys[277] upon oxidation, leading to a decrease in binding affinity (43).

As a possible oxidative sensor, LasR joins QS regulators AgrA (19) and SdiA (20), shown to have dual roles in both QS and oxidative sensing. The linking of quorum sensing to oxidative stress response through LasR may represent one of the traits that enables *P. aeruginosa* to coordinate growth and flourish under a variety of conditions (44). It is not yet clear whether redox sensing by QS regulators is important in responding to the burst of reactive oxidative species generated by the host immune system or whether this type of redox switch allows regulators to balance up-regulation of QS pathways with the potentially damaging reactive oxidative species produced through heightened metabolic activity resulting from QS activation. QS in *P. aeruginosa* has been linked to the expression of catalase and superoxide dismutase genes for protection against

H_2O_2 (45), and other recent reports provide increased evidence for the close connection between QS and central metabolism in *P. aeruginosa* (46). Although it will be interesting to know precisely how oxidative sensing by LasR might serve *P. aeruginosa*, it is perhaps more valuable to have learned more about this protein at the chemical level, noting multiple critical cysteines that may prove to be useful targets in working toward effective treatment of this pathogen.

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