Molecular Insights into Quorum Sensing in the Human Pathogen Pseudomonas aeruginosa from the Structure of the Virulence Regulator LasR Bound to Its Autoinducer*

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Matthew J. Bottomley1, Ester Muraglia, Renzo Bazzo, and Andrea Carfì
From the Istituto di Ricerche di Biologia Molecolare, Via Pontina Km 30.600, 00040 Pomezia, Rome, Italy

Many Gram-negative bacteria communicate via molecules called autoinducers to coordinate the activities of their populations. Such communication is termed quorum sensing and can regulate pathogenic virulence factor production and antimicrobial resistance. The quorum sensing system of Pseudomonas aeruginosa is currently the most intensively researched, because this bacterium is an opportunistic human pathogen annually responsible for the death of thousands of cystic fibrosis sufferers and many other immunocompromised individuals. Quorum sensing inhibitors can attenuate the pathogenicity of P. aeruginosa. Here we present the crystal structure of the P. aeruginosa LasR ligand-binding domain bound to its autoinducer 3-oxo-C12-acylhomoserine lactone. The structure is a symmetrical dimer, with each monomer exhibiting an α-β-α fold similar to the TraR and SdiA quorum sensing proteins of Agrobacterium tumefaciens and Escherichia coli. The structure was determined up to 1.8-Å resolution and reveals the atomic interactions between LasR and its autoinducer. The monomer structures of LasR, TraR, and SdiA are comparable but display differences in their quaternary organization. Inspection of their binding sites shows some unexpected variations resulting in quite different conformations of their bound autoinducers. We modeled interactions between LasR and various quorum sensing inhibitors, yielding insight into their possible mechanisms of action. The structure also provides a platform for the optimization, or de novo design, of quorum sensing inhibitors.

Evolution has endowed bacteria with a wide variety of diffusible chemical signals that are used for communication both within and between species. The most common form of intercellular communication in Gram-negative bacteria is “quorum sensing,” so named because the bacteria initiate coordinated activities only when a “quorum” population density is reached (1). This synchronization of individual behavior into cooperative group activity can have many different benefits, e.g. to produce virulence factors only when the bacterial population is sufficiently dense to survive host immune responses (2–4). The first quorum sensing system described was that of Vibrio fischeri, a symbiont of the Hawaiian bobtail squid (5, 6). The squid has a “light organ” wherein it provides nutrition for V. fischeri. Upon reaching high population density the bacteria produce bioluminescence enabling the squid to cancel its own shadow and thereby avoid predation (7). The quorum sensing mechanism triggering bioluminescence depends on the bacterial synthase LuxI, which constitutively produces a signal molecule, an acylhomoserine lactone (acyl-HSL or AHL),2 the autoinducer. The AHL diffuses through the bacterial envelope and, upon reaching a threshold concentration, the AHL binds and activates its receptor LuxR, a transcriptional activator of the luciferase operon (1). Many different Gram-negative bacteria have now been shown to use quorum sensing systems composed of LuxI/LuxR homologues and AHL autoinducers that elicit diverse responses (8). The LuxI homologues show moderate amino acid conservation (28–35% identity), whereas the LuxR homologues display only 10–25% identity.

Of the many quorum sensing systems known, that of Pseudomonas aeruginosa is the most intensively studied due to the potentially fatal effects of its infections, which kill several thousand immunocompromised individuals each year (9). P. aeruginosa is an opportunistic pathogen causing death in the majority of cystic fibrosis sufferers and in AIDS patients, burn victims, and neutropenic cancer patients (10). It is the most common Gram-negative bacterium found in hospital-acquired infections, being responsible for nosocomial pneumonia, urinary tract infections, and surgical wound or bloodstream infections (11). Current therapies lack efficacy, partly because P. aeruginosa creates and inhabits surface-associated biofilms conferring increased resistance to antibiotics and host immune responses (12). P. aeruginosa can also adapt to overcome the selective pressures imposed by growth-restricting antibiotics. Therefore, there is an urgent requirement for novel antibacterial treatments to counter P. aeruginosa. It is thus of interest that quorum sensing systems control many aspects of P. aeruginosa pathogenesis, e.g. the expression of virulence factors and a greater resistance of biofilms to antibiotics and the immune system (13–16). This presents opportunities for the design of quorum sensing inhibitors (QSIs), which reduce virulence, pathogenicity, and resistance rather than directly inhibiting...
growth, with the important ramifications that they would be unlikely to exert selective pressures leading to the emergence of drug-resistant bacteria and would not kill the beneficial gut flora (17).

In P. aeruginosa, quorum sensing of high population density induces the production of virulence factors potentially fatal to the infected patient (18, 19). The LasI synthase of P. aeruginosa constitutively produces the signal 3-oxo-C12-HSL (N-3-oxododecanoyl-L-homoserine lactone), which accumulates with population growth and activates LasR, a transcriptional regulator (R protein) homologous to LuxR. When activated by 3-oxo-C12-HSL, LasR dimers bind target gene promoters and activate the transcription of many toxic virulence factors, including exoproteases, exotoxins, and secondary metabolites (18–22). Activated LasR also participates in the maturation of biofilms, which commonly result in persistent pathogenic infections (3, 23). Overall, quorum sensing in P. aeruginosa probably controls over 350 genes, of which ~30% encode virulence factors (16, 24).

Evidence now shows that QSIs targeting LasR can attenuate the pathogenicity of P. aeruginosa (17, 25, 26). QSI compounds have been obtained from natural sources or by synthesis of AHL analogues or by screening random libraries (reviewed in Ref. 16), but so far none of these are suitable for medical application. Therefore, we sought to aid the rational development and/or optimization of QSIs by generating molecular information on the uppermost protein in the hierarchy of quorum sensing regulators in P. aeruginosa, namely LasR (2, 17, 19, 27–30). Here we present the structure of LasR in complex with its autoinducer and interpret this structure in the context of the activation and inhibition of quorum sensing.

EXPERIMENTAL PROCEDURES

Sample Preparation—Using a P. aeruginosa cosmid from the Pseudomonas Genetic Stock Center (Greenville, NC), full-length LasR (GenBank™ D30813) was PCR-cloned into a pETM-11 vector (EMBL Heidelberg). When expressed in Escherichia coli, either in the presence or absence of 3-oxo-C12-HSL, full-length LasR (239 residues) was largely insoluble and was resistant to refolding. Subsequently, numerous shorter LasR constructs were cloned and tested for expression. A construct spanning Met-1 to Lys-173, the predicted ligand-binding domain (LBD), was highly soluble in the presence of 3-oxo-C12-HSL. The TraR LBD (Met-1 to Thr-165) was cloned from an existing in-house plasmid (31).

In 1 liter of rich LB medium with induction by 0.4 mM isopropyl-β-D-galactopyranoside at 23 °C for 18 h, LasR-LBD (GenBank™ D30813) was PCR-cloned into a pETM-11 vector, in the presence or absence of 3-oxo-C12-HSL. The molecular mass of the purified LasR-LBD protein (19,432 Da per monomer) and the presence of 3-oxo-C12-HSL (C12H27NO4, 298 Da) in a buffer-exchanged sample lacking excess AHL were confirmed by mass spectrometry.

Impact of AHLs and QSIs on Protein Solubility—LasR-LBD and TraR-LBD proteins were produced in E. coli from the tightly regulated pETM-11 vector, in the presence or absence of 10 μM AHLs or QSIs. Expression was induced at 21 °C at an optical density (A600) of 0.4 unit using 0.2 mM isopropyl-β-D-galactopyranoside. To account for any variable effects of the AHLs or QSIs on overall cell growth and/or protein synthesis, cell cultures were harvested not after a predetermined time but rather only when the A600 nm had reached 1.4 units, typically 4–6 h. Cells were disrupted by microfluidization in lysis buffer: 40 mM Tris-HCl, pH 8.0, 200 mM NaCl, 5 mM imidazole, 2 mM mercaptoethanol, 0.5% (w/v) glycerol, 0.01% (w/v) Nonidet P-40 detergent, and a protease inhibitor mixture. The lysate was centrifuged at 12,000 rpm for 30 min. The soluble supernatants were purified by Ni2+ affinity chromatography. The insoluble pellets were resuspended in lysis buffer plus 2% SDS detergent. Normalized sample volumes were examined by SDS-PAGE.

Crystallization and X-ray Data Collection of LasR-LBD in Complex with 3-Oxo-C12-HSL—Single LasR-LBD crystals were obtained from 10 mg/ml stocks of native or selenomethionine derivative protein using the hanging-drop vapor diffusion method. Crystals grew after 1–2 days equilibration at 18 °C against a reservoir of 20% w/v polyethylene glycol 4000, 80 mM calcium acetate, 40 mM Hepes, pH 7.3, 5 mM dithiothreitol, and 0.5 mM 3-oxo-C12-HSL. X-ray diffraction data were collected from flash-frozen crystals in a 100 K nitrogen cryostream. Three cycles of crystal annealing by the flash-annealing method (34) improved the diffraction quality.

Structure Determination and Refinement—We were unable to solve the x-ray data from native LasR-LBD crystals by molecular replacement using the known TraR structure as a search model (it has only 16% sequence identity). Thus, a single anomalous dispersion experiment was performed with the x-ray wavelength tuned to the selenium peak (0.980 Å) determined from an x-ray fluorescence scan (beamline BW7A, DESY, EMBL Hamburg). Diffraction images were indexed and integrated in the P21 space group using MOSFLM. The data were consistent with a unit cell containing two LasR-LBD dimers and 42% solvent. Each native LasR-LBD monomer contains four methionine residues (excluding the N-terminal methionine), and consequently 16 selenomethionine sites were sought using CNX (Accelrys Software Inc.). The Patterson maps obtained revealed peaks for all 16 selenomethionine sites, which were refined and used for phase calculation and density modification, yielding a clearly interpretable electron density map. Further density modification, solvent flattening, and phase extension to 1.8 Å provided a map that was auto-traced to 80% completeness by ARP/wARP (35). Model building was per-
LasR-Autoinducer Crystal Structure from P. aeruginosa

formed using O (36) and refinement using REFMAC (37), defining one TLS group per protein chain.

Structure Analysis and Modeling of Interactions with QSIs—The structural quality was analyzed using the Molprobity server (38). Structural alignments of LasR, TraR, and SdiA were performed using DALI (39). Protein-ligand images were prepared with PyMOL. 3

For modeling the LasR-QSI interactions, hydrogen atoms were added to LasR to simulate a pH of 7.4. The positions of these hydrogens and of the protein side chains were optimized by energy minimization (5000 steepest descent steps), using the Merck molecular force field (MMFF-S) as implemented in Macromodel version 9.0 (Schrodinger LLC software), keeping first the protein backbone and then the AHL structure rigid. Subsequently, the AHL geometry was allowed to relax while keeping the protein rigid. Each QSI, except TP-1 and TP-5, was manually docked into the binding site by superimposing the key atomic fragments onto the AHL lactone ring and amide group. Because the binding pocket is buried, it was not possible to perform automated surface docking. The LasR protein structure was restrained as a rigid body, and the position of the QSI was constrained to a conjugate gradient energy minimization until convergence was reached. In the case of TP-1 and TP-5, 500 conformers, generated by Monte Carlo type conformational analysis, were docked into LasR and submitted to energy minimization as above. The structures represent the LasR-QSI complexes with the lowest energy values.

RESULTS

Purification of Soluble LasR—Despite the medical significance of LasR, there are very few reports describing its biochemical or biophysical characterization, presumably due to the noted difficulty of purifying soluble LasR (41) and the need to synthesize its AHL. Therefore, we cloned differently sized LasR proteins and screened for useful expression constructs. We found one C-terminally truncated construct that enabled the production of large amounts of soluble LasR protein. Indicative of functional integrity, this LasR protein was soluble and stable only if produced in the presence of its cognate AHL ligand, 3-oxo-C12-HSL. The dependence on AHL for solubility is similar to that reported for full-length TraR (42), and was also observed herein for a ligand-binding domain construct of TraR (Fig. 1). Decreasing amounts of the LasR protein were soluble if produced in the presence of one of the following AHLs: 3-oxo-C8-HSL, 3-oxo-C6-HSL, and 3-oxo-C12-HSL, the autoinducers of TraR, LuxR, and RhlR, respectively (Fig. 1). Despite the apparent similarities of these quorum sensing systems, none of the non-cognate AHLs formed stable complexes with LasR, i.e. they had all precipitated 1–2 h post-purification. This lack of promiscuity by LasR agrees with previous findings that the autoinducers of LasR and LuxR show virtually no cross-functionality (43). The soluble LasR construct (hereafter termed LasR-LBD) spanned the predicted LBD and oligomerization domain and lacked the C-terminal 60 residues of the DNA-binding domain.

Structure Determination of the LasR-AHL Complex—The soluble LasR-LBD-3-oxo-C12-HSL complex was purified to homogeneity and crystallized. We solved the structure via the Molprobtity server (38). Structural alignments of LasR, TraR, and SdiA were performed using DALI (39). Protein-ligand images were prepared with PyMOL. 3

| TABLE 1 | Data collection and refinement statistics for LasR-LBD complexed with 3-oxo-C12-HSL |
| :--- | :--- |
| **Data collection statistics** |  |
| Space group | P21 |
| Cell dimensions (/) | a = 53.82, b = 85.33, c = 75.51 |
| Cell angles | α = γ = 90°, β = 95.8° |
| Solvent content in unit cell | 45% |
| Resolution range (/) | 40.0–1.80 |
| Number of unique reflections* | 61,495 (8,825) |
| Multiplicity* | 8.1 (7.5) |
| Completeness* | 97.8 (96.9) |
| Mean I/S.D.** | 26.4 (3.1) |
| Rfree* | 9.0 (26.3) |

**Refinement statistics**

| Rfree (%) of total reflections | 25.4 |
| No. reflections in Rfree set | 3,112 (218) |
| Protein residues§ | 660 |
| Protein atoms (non-hydrogen) | 5,140 |
| Ligand atoms (non-hydrogen) | 84 |
| Water molecules§ | 542 |

**Quality of structure**

| r.m.s.d. bond lengths (/) | 0.007 |
| r.m.s.d. bond angles (degrees) | 1.10 |
| B protein main chain* | 3.63 |
| B protein side chains | 4.65 |
| B water molecules | 2.82 |
| B water molecules | 15.48 |

*Values for the outermost resolution shell spanning 1.9-1.8 Å are in parentheses.
§ The purified LasR-LBD was 175 residues, but for each chain some of the following residues were not visible in the electron density maps: the N-terminal Gly-Ala tag, Met-1 to Asp-5, and His-169 to Lys-173.
§ Only includes water molecules visible above 1.1σ in the 2Fo–Fo electron density map.
¶ Average B-factor for specified set after TLS refinement.

3 W. L. DeLano (2002) PyMOL, DeLano Scientific, San Carlos, CA.
The biologically relevant complex in the unit cell is a symmetrical dimer of LasR-LBDs, with each monomer containing one deeply buried ligand (Fig. 2). The monomer fold is an α-β-α sandwich with three α-helices packed on both sides of a five-stranded anti-parallel β-sheet. The 3-oxo-C_{12}-HSL lies parallel to the β-sheet and is buried from the solvent in a pocket formed between the β-sheet and helices α3, α4, and α5. On the other side of the β-sheet, helix α6 makes the majority of the intermolecular H-bonds and hydrophobic contacts contributing to the formation of a large dimer interface burying ~1900 Å² of surface area. According to dynamic light scattering, analytical gel filtration, and NMR spectroscopy (data not shown), the LasR-LBD is a constitutive dimer in solution, presumably enabling the binding in vivo of full-length LasR to promoters with two DNA target motifs (19, 21, 22).

Analysis of the AHL Binding Site in LasR—The LasR-LBD structure reveals the atomic interactions between LasR and its autoinducer. All the polar groups of 3-oxo-C_{12}-HSL, except the lactone ring oxygen, make H-bonds with the LasR-LBD. A total of six intermolecular H-bonds were observed: five direct and one water-mediated, involving Tyr-56, Trp-60, Arg-61, Asp-73, Thr-75, and Ser-129 (Fig. 3). In particular Tyr-56, Trp-60, Asp-73, and Ser-129 are strongly conserved in LuxR homologues, reflecting the shared activation mechanism by AHLs with identical HSL headgroups (Fig. 1B). In contrast with this conservation, the long acyl chain extends into a cavity lined with hydrophobic residues, some of which are seen only in LasR and not in other LuxR homologues, e.g. Leu-40, Tyr-47, Cys-79, and Thr-80 (Fig. 4C). These non-conserved residues contact the extreme methylene/methyl groups of the acyl chain, which are absent from the shorter AHL ligands of most LuxR homologues. The tight encapsulation of the AHL by LasR means that its function can be abolished even by relatively conservative point mutations in the AHL binding pocket, e.g. Y64H, A70E, D73E, P74L, and W88Y (21, 44). These structural features presumably provide high ligand specificity and minimize cross-talk between different bacterial species.

Structural Comparisons of LasR: The Overall Fold—Despite low amino acid sequence identities (10–16%), the LasR-LBD monomer shows considerable structural similarity to the quorum sensing proteins TraR from the plant pathogen Agrobacterium tumefaciens (31, 45) and SdiA from E. coli (46), and to a lesser extent to the GAF and PAS domains found in mammalian signaling pathways (47). The similarities between LasR, TraR, and SdiA are both structural and functional, because all three bind AHLs in a binding site that is conserved between the β-sheet and the external α-helical face made of α3, α4, and α5. Like LasR, TraR governs bacterial pathogenicity. 3-Oxo-C_{8}-HSL binding to TraR activates quorum sensing in A. tumefaciens, which then induces tumors on plant hosts (48). Although relatively little is known about SdiA, it can bind several different AHLs with 8-carbon acyl chains and is the only LuxR homologue in E. coli (46). Because E. coli lacks an endogenous AHL synthase, it has been suggested that SdiA may allow E. coli to sense and respond to the presence of diverse bacterial species.

The ligand-bound LasR, TraR, and SdiA-LBDs have a shared monomer topology with pairwise Ca r.m.s.d. values of ~2.4 Å over 150 residues (Fig. 4). Both LasR and TraR exist as dimers, whereas SdiA-LBD is monomeric. The largest conformational differences between the LasR and SdiA monomers occur in

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**FIGURE 2.** Structural features of LasR-LBD. A ribbon representation of dimeric LasR-LBD showing each protein monomer (blue and cyan) with a buried 3-oxo-C_{12}-HSL autoinducer (yellow, carbon; blue, nitrogen; and red, oxygen). The internal α-helical face, comprising α4 and α6, makes many intermolecular interactions stabilizing the dimer. The two monomers are symmetry-related by a 180° γ-axis rotation. The coordinates have been deposited in the Protein Data Bank (entry code 2UV0).

**FIGURE 3.** The AHL binding site in LasR. A, LasR side chains make many H-bonds (dotted lines) to the HSL of the autoinducer 3-oxo-C_{12}-HSL. The binding network involves (i) Tyr-56-OH with the amide 1-oxo (2.65 Å), (ii) likewise Ser-129-OG with the 1-oxo (2.70 Å), (iii) Trp-60-NE with the lactone carbonyl (3.01 Å), (iv) Asp-73-OD2 with the amide NH (2.76 Å), (v) Thr-75-OG1 likewise (3.39 Å), and (vi) an indirect H-bond between the 3-oxo group and a water molecule (2.85 Å) and then Arg-61 NE1 (2.90 Å) and NH1 (2.84 Å). The conformation of Asp-73 is stabilized by an H-bond with Tyr64. The 12-carbon AHL is housed in a large hydrophobic pocket formed by residues Leu-36, Gly-38, Leu-39, Leu-40, Tyr-47, Glu-48, Ala-50, Ile-52, Tyr-56, Trp-60, Arg-61, Tyr-64, Asp-65, Gly-68, Tyr-69, Ala-70, Asp-73, Pro-74, Thr-75, Val-76, Cys-79, Thr-80, Trp-88, Tyr-93, Phe-101, Phe-102, Ala-105, Leu-110, Thr-115, Leu-125, Gly-126, Ala-127, and Ser-129; many of these are conserved in TraR and SdiA, as shown in Fig. 4C. The distances quoted are between the heavy atoms mediating the H-bonds and are

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**LasR-Autoinducer Crystal Structure from P. aeruginosa**
majority of dimer-stabilizing intermolecular bonds occur between the $\alpha_6$ helices. However, in the TraR dimer the $\alpha_6$ helices are almost parallel, whereas in LasR dimers these helices are perpendicular. Consequently, LasR and TraR dimers cannot be closely superimposed due to a $90^\circ$ difference of monomer-monomer orientation (Fig. 4B, right).

**Structural Comparisons of LasR:**

*The Ligand Binding Site—Structural comparisons of LasR, TraR, and SdiA further clarify the atomic basis for the specificity of LasR for the *P. aeruginosa* autoinducer $3\text{-oxo-C}_{12}$-HSL. As described above, the AHL binding pocket of LasR contains several residues that are structurally and functionally conserved in most LuxR homologues, including TraR and to a lesser extent SdiA (Fig. 4C). Such high conservation reflects the invariant nature of the HSL moiety in AHL ligands. However, although many of the amino acids binding the HSL moiety are structurally conserved in LuxR homologues, there are some surprising differences in the H-bonding network through which these residues coordinate the AHLS in LasR and TraR. In the LasR complex, the 1-oxo group of the HSL forms direct H-bonds to both Tyr-56 and Ser-129, whereas the 3-oxo group H-bonds via a water molecule to the Arg-61 side chain (Figs. 3 and 5). In contrast, in the TraR complex, the HSL 1-oxo is bound only by Tyr-53 (2.79 Å) and not by the more distant Thr-129 (4.18 Å). Furthermore, in the TraR complex, the 3-oxo H-bonds via a water molecule to both the Thr-129 side chain and the backbone carbonyl of Ala-38 (Fig. 5). The residue equivalent to LasR Arg-61 is TraR Gln-58, but its side chain points away from the HSL, excluding the possibility of binding to the 3-oxo of the TraR ligand. The residue structurally equivalent to TraR Ala-38 is LasR Leu-36, which does not H-bond with the ligand but instead forms an intramolecular H-bond with the adjacent $\beta$-strand. Thus, a somewhat paradoxical situation is observed between LasR and TraR, where spatially comparable residues exhibit different functionalities within the conserved context of AHL binding (Fig. 5).

In addition to the subtle differences in H-bonding to the HSL moiety, or perhaps as a consequence, the acyl chains of the LasR and TraR ligands adopt highly different conformations.

FIGURE 4. Overall fold comparison of LasR, TraR, and SdiA. A, ribbon diagrams of superposed LasR (blue) and SdiA (pink) monomers (SdiA coordinates from PDB entry 2AVX). The orientation of LasR is similar to that in Fig. 2. B, ribbon representations of superposed LasR and TraR. The LBD monomers on the left (blue, LasR; green, TraR) have been superimposed, clearly revealing the structural similarity of their folds (r.m.s.d. 2.4 Å for 150 residues). However, the overall structural differences of the dimers are revealed by the monomers on the right (cyan, LasR; red, TraR), due to their different dimer interfaces, they are rotated −90° with respect to each other such that their $\beta$-sheets are roughly perpendicular. C, a structure-based sequence alignment of LasR, TraR, and SdiA. The major secondary structure elements of LasR and TraR are shown above and below the alignment, respectively; they are also conserved in SdiA. Asterisks indicate the residues making H-bonds to the AHLS; hashes indicate additional residues forming the ligand-binding pockets of LasR (above) and TraR (below). Numbering is for LasR.
LasR-Autoinducer Crystal Structure from P. aeruginosa

Although both AHLs are bound parallel to the plane of the β-sheet, the acyl chains curve away from the HSL binding site in opposite directions, starting from the 3-oxo position (Fig. 5). Thus, the LBDs show remarkable structural plasticity whereby two very similar ligands can be accommodated in different conformations within the same site of the same overall scaffold.

Although the biological ligand of SdiA is not known, the SdiA-LBD structure has been solved in complex with C8-HSL, which lacks the 3-oxo group of most AHLs. As in LasR and TraR, the C8-HSL is deeply buried in SdiA between the β-sheet and helices α3, α4, and a short ζ10 helix. However, in contrast with the AHLs clearly observed in the LasR and TraR crystal structures, the C8-HSL in the SdiA NMR structure ensemble does not have a well defined position (46). This uncertainty results from a paucity of nuclear Overhauser effects between SdiA and the AHL and is attributable to conformational heterogeneity of the ligand, thus precluding an atomic understanding of the interaction mechanism. Nevertheless, the residues implicated in AHL binding due to their proximity (Tyr-63, Trp-67, Tyr-71, Asp-80, and Trp-95) are positionally well defined, and these are conserved in LasR and TraR (Fig. 4C).

Quorum Sensing Inhibition via LasR—The emergence of LasR as a key regulator of P. aeruginosa pathogenesis has made its inhibition an interesting pharmaceutical goal, and a few QSIs have already been reported (16). We attempted to study the interactions of LasR with the commercially available QSIs: patulin and penicillic acid (Fig. 6A), which were both identified by screening extracts of Penicillium fungi (55) and also with the brominated furanone C30 (Fig. 6A), kindly provided by Prof. M. Givskov and coworkers (25). Using NMR spectroscopy, which can detect even very weak protein-ligand interactions, we could not detect any binding of patulin, penicillic acid, or C30 to the LasR-LBD-AHL complex (data not shown). We conclude that, once bound, the AHL is so deeply and tightly buried that it cannot be displaced by these QSIs. Further, there are no alternative sites for allosteric interactions of the QSIs on the surface of LasR. Therefore, we attempted to prepare LasR-LBD-QSI complexes by producing LasR in E. coli in the absence of AHL and the presence of QSIs. None of the inhibitors formed a soluble complex with LasR. We conclude that the QSIs probably act by competing with AHLs for binding to the nascent polypeptide and inhibiting the correct folding, solubility, and/or stability of the wild-type protein. We then modeled the interactions of QSIs with the LasR-LBD in silico and interpreted these models with respect to the regulation of quorum sensing.

The first QSIs discovered were secondary metabolites of the Australian seaweed Delisea pulchra, which uses QSIs to protect its surface from colonization by marine bacteria (49). These QSIs are halogenated furanones resembling AHLs (Fig. 6A, compound 2) and are weak inhibitors of quorum sensing in P. aeruginosa (50). However, combinatorial chemistry has led to the discovery of improved brominated furanone QSIs, e.g. C30, which inhibits LasR and many quorum sensing-controlled genes (Fig. 6A, compound 3) (25). Indeed, in experimental models, C30 can reduce virulence factor production, interfere with biofilm formation, increase susceptibility to antibiotics, and promote clearance of infections by the immune response, without directly restraining bacterial growth (25). However, the use C30 is compromised by its instability in vivo (16). A model of LasR complexed with a halogenated furanone reveals how the furanone is likely to bind LasR similarly to the lactone ring of 3-oxo-C12-HSL, i.e. packing against numerous aromatic side chains and H-bonding with Trp-60 (Fig. 6B). However, the absence of the long acyl chain in either the algal furanone or C30, and the inability to H-bond with Asp-73 or Thr-75, would prevent the correct formation of the hydrophobic core of LasR, because the acyl chain of the natural AHL mediates the packing of helices α3, α4, and α5 onto the central β-sheet. The absence of important fold-promoting features would result in an unstable protein and the observed QSI activity. Although the halogens are lipophilic and therefore suited to the hydrophobic ligand binding pocket, it is noteworthy that one of the bromines clashes with a water molecule mediating an H-bond between the side chain of Tyr-93 and the carbonyl of Leu-110 (Fig. 6B). The breaking of an internal H-bond by the QSIs would also destabilize the protein core. Indeed, it has been noted previously that generally, subtle modifications at the same lactone carbon-4 position (Fig. 6A) produce LasR antagonists, whereas those at the 3-position produce agonists (51). These observations may indicate that the environment around the 3-position, composed of several potentially mobile protein side chains, is relatively permissive and might be exploitable in the future for the structure-based design of improved QSIs. In contrast, the 4-position may be more sensitive because it abuts residues undergoing structurally important interactions (Tyr-93 with Leu-110) and is more closely packed against Trp-88, a conserved element of R protein binding pockets (Fig. 4C).

Naturally occurring QSIs have also been discovered in plant, fungi, and animal extracts (52–54). To date the most potent such QSIs are patulin and penicillic acid, but both are mycotox-
ins and thus unsuitable for clinical use. Promising QSI activities have also been found in extracts of carrot, habanero chili, water lily, bee propolis, and garlic (55), but the active QSI components therein are currently unknown. A model of the LasR-patulin complex suggests that patulin mimics the lactone of AHLs and probably forms the canonical H-bond with Trp-60 (Fig. 6C). Patulin may additionally bind LasR via H-bonds between (i) the oxygen of its six-member ring and the Tyr-93 side chain, and (ii) its hydroxyl group and the Asp-73 or Thr-75 side chain. However, as for the halogenated furanones, the absence of an acyl chain would prevent formation of the hydrophobic core of LasR and result in the destabilizing QSI activity.

An alternative route to QSI discovery is the design of inhibitors that are structurally similar to 3-oxo-C12-HSL. Here it is less simple to propose an inhibitory mechanism, because only subtle changes are made to the lactone while maintaining the 12-carbon chain. However, modeling of LasR complexes with an agonist and a related antagonist (Fig. 6A, compounds 5 and 6) (41) provides some molecular insight. The agonist can clearly mimic the natural AHL, and it is not surprising that compound 5 is a LasR activator (Fig. 6D). In contrast, although the antagonist can also be readily modeled in the same site, its planar phenyl ring would have to adopt an unfavorable conformation perpendicular to its amide group. Because these moieties would have a lower energy in the coplanar conformation, one can speculate that the rearrangement required from the coplanar solution conformation to the perpendicular bound form would require a relatively large input of energy. A decreased on-rate and the consequent stalling of protein folding might underlie its QSI activity.

A comprehensive approach toward the generation of LasR inhibitors would benefit from the investigation of additional different (non-AHL resembling) classes of compounds. Such efforts are exemplified by the recent identification of the specific LasR agonist TP-1, a tri-phenyl structure (Fig. 6A, compound 7) (56). To understand the mechanism of TP-1, Mühl et al. (56) made a homology model of LasR based on the TraR/AHL structure and used this to derive a model of the LasR-TP-1 complex. However, as described above (Fig. 5A), the structural differences in the binding pockets of TraR and LasR mean that
a TraR-based LasR homology model is inappropriate, probably resulting in the erroneous placement of TP-1 in the model of Müh et al. When modeled with the crystal structure of LasR determined here, the position of TP-1 in the binding site is quite different (Fig. 6E). In contrast with the complex of Müh et al., the amide group of TP-1 was not readily placed in the vicinity of Asp-73 or Thr-75, suggesting that this H-bond does not occur. Nevertheless, the formation of H-bonds with Tyr-56, Trp-60, and Ser-129, and the filling of the entire ligand binding pocket by the extended triphenyl compound, strongly suggest why TP-1 is a good agonist of LasR. In contrast with TP-1, the related compound TP-5 (Fig. 6A, compound 8) is a quorum sensing inhibitor. Unlike TP-1, all three phenyl rings of TP-5 cannot be simultaneously fitted into the LasR binding site due to its reduced degrees of rotational freedom imposed by the loss of a methylene group when compared with TP-1 (highlighted with an asterisk in Fig. 6A). The partial binding of TP-5 without the ability to correctly pack into the protein core would explain its QSI activity.

**DISCUSSION**

The model for LasR activation that emerges from our structure requires the binding of 3-oxo-C₁₂-HSL to the nascent protein, with the ligand stabilizing the protein fold both via van der Waals’ interactions within a hydrophobic core and via H-bonded linking of distal elements of secondary structure (e.g. the AHL 1-oxo group links Tyr-56 in the β2–α3 loop to Ser-129 of strand β3). Concomitant with folding, LasR dimerization can occur, enabling binding of promoter DNA and transcriptional activation of quorum sensing controlled genes.

The AHL-dependent folding switch of LasR is probably representative of quorum sensing regulation in all LuxR homologues, but the R protein structures compared above reveal several differences in their mechanisms. Notably, all three regions of difference between the LasR-LBD and SdiA-LBD structures are involved in the dimerization of LasR-LBD. Because SdiA-LBD is monomeric, it is likely that these structural differences account for the different oligomeric states of the two proteins, which may create differences in the range of target promoter sequences they can recognize. Similarly, although TraR binds only palindromic DNA target sequences, the different arrangement of the LasR dimer may facilitate binding to a less restricted set of sequences in its ~350 target genes. Indeed, an extensive study of LasR interactions with Las-responsive promoters revealed high affinity binding of LasR both to palindromic and non-palindromic sites, i.e. dyad symmetry was not a specific sequence requirement (22), suggesting a more versatile nature of LasR compared with TraR.

The detailed analysis of the AHL-binding mechanism of LasR, and its comparison with TraR and SdiA, suggests that the ligand-binding scaffold has evolved differently to suit its hosts. Both LasR and TraR have binding pockets precisely adapted to the size of their ligands, a bigger binding pocket is formed in LasR (~670 Å³) compared with TraR (~440 Å³), to favor specifically the accommodation of the C₁₂-AHL ligand made by P. aeruginosa. In contrast, the more spacious, less tightly packed, accommodation of C₆-HSL in the SdiA-LBD (pocket volume ~780 Å³) is presumably what enables SdiA to bind various ligands, including 3-oxo-C₉-HSL and C₇-HSL, without major structural rearrangement (46). This highlights the functional differences of the quorum sensing systems in the different bacterial species. SdiA of E. coli can bind various AHLs from other species and may thus represent a signal interception mechanism, rather than a canonical quorum sensing system. Such a system seems suitable for the enteric growth environment of E. coli, which competes with large populations of diverse bacterial species. In contrast, the free-living bacteria (P. aeruginosa and A. tumefaciens) often inhabit sparser environments where the most important communication is intra-species.

Although AHLs and their cognate R proteins thus appear to have evolved complementarily to suit diverse bacterial needs, some eukaryotic species have evolved with the ability to produce protective compounds able to interfere with bacterial quorum sensing. Our efforts to understand the molecular mechanisms of quorum sensing systems led us to model the interactions of such QSIs with LasR. Comparing QSIs with natural AHLs revealed considerable structural and functional similarity between their putative interactions with LasR.

To summarize, the structure described herein yields insight into both the natural functioning of the quorum sensing system of P. aeruginosa and into the molecular mechanisms of some recently identified QSIs. The QSIs discussed, in particular C₃₀, include novel anti-pathogenic agents that attenuate bacterial virulence and increase susceptibility to biocide treatment. Although these compounds are not currently of clinical use, they suggest that QSIs hold therapeutic potential. However, one potential limitation of such QSIs is raised in a recent report of a pro-apoptotic immunomodulatory effect of 3-oxo-C₁₂-HSL on host cells (40). Although the true impact of AHLs on host physiology in vivo has yet to be established, the potential off-target effects of agonist-like QSIs highlight the importance of the continued search for novel structural classes of QSIs. We anticipate that the structure and analysis presented herein will greatly facilitate the progress of the rational development and optimization of novel QSIs that target LasR to combat this important human pathogen.

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LasR-Autoinducer Crystal Structure from P. aeruginosa

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