Negative Regulation of Quorum-Sensing Systems in *Pseudomonas aeruginosa* by ATP-Dependent Lon Protease[▽]

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Lon protease, a member of the ATP-dependent protease family, regulates numerous cellular systems by degrading specific substrates. Here, we demonstrate that Lon is involved in the regulation of quorum-sensing (QS) signaling systems in *Pseudomonas aeruginosa*, an opportunistic human pathogen. The organism has two acyl-homoserine lactone (HSL)-mediated QS systems, LasR/LasI and RhlR/RhlI. Many reports have demonstrated that these two systems are regulated and interconnected by global regulators. We found that *lon*-disrupted cells overproduce pyocyanin, the biosynthesis of which depends on the RhlR/RhlI system, and show increased levels of a transcriptional regulator, RhlR. The QS systems are organized hierarchically: the RhlR/RhlI system is subordinate to LasR/LasI. To elucidate the mechanism by which Lon negatively regulates RhlR/RhlI, we examined the effect of *lon* disruption on the LasR/LasI system. We found that Lon represses the expression of LasR/LasI by degrading LasI, an HSL synthase, leading to negative regulation of the RhlR/RhlI system. RhlR/RhlI was also shown to be regulated by Lon independently of LasR/LasI via regulation of RhlI, an HSL synthase. In view of these findings, it is suggested that Lon protease is a powerful negative regulator of both HSL-mediated QS systems in *P. aeruginosa*.

Proteolysis in bacteria is important for maintaining cellular homeostasis. Most intracellular proteolysis is initiated by ATP-dependent proteases, including Lon, FtsH, ClpAP, ClpXP, and HslVU, which have been identified in *Escherichia coli* (8, 11, 18). These proteases harbor a domain conferring ATPase activity, characteristic of the ATPase family associated with diverse cellular activities (AAA⁺) (16). They are involved in protein quality control by degrading misfolded and denatured proteins. Among them, Lon and ClpXP are responsible for 70 to 80% of the energy-dependent degradation of proteins in vivo (22, 27). Furthermore, they perform important regulatory functions in bacterial cells by controlling the availability of critical regulatory proteins.

Lon, first identified in *E. coli*, has been shown to degrade specific regulatory proteins involved in a variety of biological processes: SulA, which regulates cell division (29); RcsA, a transcriptional activator for capsule synthesis (41); λ N, which antagonizes the termination of early transcription of λ DNA (26); and CcdA, the antitoxin of the *ccd* postsegregational killing system carried by the F plasmid (42). Lon has been shown to regulate virulence factors in some pathogenic bacteria. For instance, it specifically degrades HilC and HilD, which are transcriptional regulators for the expression of *Salmonella* pathogenicity island 1 in *Salmonella enterica* serovar Typhimurium (38). It recognizes YmoA, which regulates the Yop regulon in *Yersinia pestis* (17). Furthermore, it is involved in

degrading HrpR, which regulates the expression of the Hrp system in *Pseudomonas syringae* (4). Proteolysis of these regulators by Lon is important for pathogenesis. In fact, we have previously demonstrated that it is essential for systemic infection with serovar Typhimurium in mice (39).

Pseudomonas aeruginosa is an opportunistic human pathogen that tends to infect individuals with cystic fibrosis or immunocompromised patients, such as those suffering burns or undergoing cytotoxic chemotherapy (24). A variety of virulence factors have been reported in *P. aeruginosa*, e.g., proteases, including elastase, alkaline protease, LasA protease, protease IV, and membrane protease (25). They interfere with the host immune response by degrading target cell components. In addition, extracellular virulence factors, such as pyocyanin, exotoxin, hemolysin, and rhamnolipids, are involved in the expression of *P. aeruginosa* virulence (5). In most cases, synthesis of these factors is controlled by a quorum-sensing (QS) system.

A QS system is a regulatory mechanism that allows bacteria to monitor their population size by responding to the extracellular concentration of a signal molecule. *P. aeruginosa* has two QS systems mediated by acyl-homoserine lactone (HSL) as the signal molecule. They are encoded by the *lasR-lasI* and *rhlR-rhlI* gene pairs. *lasI* and *rhlI* encode HSL synthases (LasI and RhlI), which are responsible for the synthesis of 3-oxo-C₁₂-HSL and C₄-HSL, respectively. *lasR* and *rhlR* encode the transcriptional activators (LasR and RhlR) that respond to their cognate signal molecules and activate transcription of *lasI* and *rhlI*, respectively. The two systems function sequentially: the RhlR/RhlI system is subordinate to the LasR/LasI system, because *rhlI* and *rhlR* are among the genes activated by LasR and LasI. The QS systems control over 200 genes, including

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[▽] Published ahead of print on 11 April 2008.

TABLE 1. Bacterial strains used in this study

Strain	Relevant properties ^a	Reference or source
<i>P. aeruginosa</i>		
PAO1	Prototroph	15
CS9008	$\Delta lon::Gm$ in PAO1	This study
CS9011	$\Delta rhlR$ in PAO1	This study
CS9013	$\Delta lasR$ in PAO1	This study
CS9027	$\Delta lon::Gm$ in CS9013	This study
CS9028	$\Delta lon::Gm$ in CS9011	This study
CS9038	$\Delta lasI$ in PAO1	This study
CS9044	$\Delta rhlI$ in PAO1	This study
CS9051	$\Delta rhlI$ in CS9013	This study
CS9053	CS9008 harboring pTKY805	This study
CS9062	$\Delta lon::Gm$ in CS9051	This study
<i>E. coli</i>		
CS5485	DH5 α Z1 harboring pTKY715	This study
CS5487	DH5 α Z1 harboring pTKY714	This study
DH5 α Z1	DH5 α <i>lacI</i> ^q	Our collection
SM10(λ pir)	<i>thi thr leu tonA lacY supE recA::RP4-2Tc::MuKm</i> λ pir	Our collection
<i>C. violaceum</i>		
CV026	Mini-Tn5 mutant of ATCC 31532	23

^a Gm, gentamicin resistance.

those for the pathogenesis of *P. aeruginosa* infections (36, 44). The *P. aeruginosa* QS system is connected in complicated ways with other cellular regulatory networks. For instance, it is regulated by functions such as Vfr (1), GacA (32), RpoS (46), and RpoN (40).

In this study, we provide the first evidence that the Lon protease of *P. aeruginosa* is involved in negative regulation of the LasR/LasI and RhlR/RhII QS systems. We identified a *lon* homologue in *P. aeruginosa* PAO1 and constructed a *lon* insertion mutant and characterized it. By these means, we have found that *lon* disruption results in LasR/LasI-dependent ac-

tivation of the RhlR/RhII system. We demonstrate that Lon regulates the expression of LasR/LasI by posttranslational control of LasI. It also regulates the RhlR level independently of LasR/LasI. We also suggest that Lon is involved in the regulation of RhlR through modulation of RhII.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are shown in Table 1 and Table 2, respectively. Bacteria were routinely grown in L broth (1% Bacto tryptone [Difco]-0.5% Bacto yeast extract [Difco]-0.5% sodium chloride, pH 7.4) and L agar at 37°C. When necessary, the medium was supplemented with gentamicin (25 μ g ml⁻¹), carbenicillin (100 μ g ml⁻¹), or ampicillin (25 μ g ml⁻¹).

Construction of plasmids. Plasmids pTKY714 and pTKY715, encoding N-terminally His-tagged *rhlR* and N-terminally His-tagged *rhlI*, respectively, were constructed by amplifying a BglII-HindIII fragment carrying *rhlR* with the primers His-*rhlRF* (5'-GGGGAAGCTTAAAGGAGGATGGAC-3') and His-*rhlRR* (5'-GGCTTAGATCATGAGGATG-3') and a BamHI-HindIII fragment carrying *rhlI* with the primers His-*rhlIF* (5'-GACGGATCCATGATCAATGC-3') and His-*rhlIR* (5'-TCGGAAGCTTCGGCCAAATCC-3') by PCR, after which the fragment was cloned into pUHE212-1.

Plasmid pTKY805 for complementation analysis of *lon* was constructed by cloning in pME6032 a 2,676-bp PCR fragment, bp -196 to +2480 relative to the *lon* translational start site.

Construction of $\Delta lon::Gm$, $\Delta rhlR$, $\Delta lon::Gm \Delta rhlR$, $\Delta lasR$, $\Delta lon::Gm \Delta lasR$, $\Delta lasI$, $\Delta rhlI$, $\Delta lasI \Delta rhlI$, and $\Delta lon::Gm \Delta lasI \Delta rhlI$ mutants. To construct the $\Delta rhlR$ mutant CS9011, the plasmid pTKY714 was cleaved at the two NruI sites to eliminate the 214-bp central fragment of *rhlR*. The resultant plasmid, pTKY762, was cleaved at the EcoRI and HindIII sites in the vector, and the *rhlR*-disrupted fragment was ligated to the vector pEX18, a transferable suicide vector (14), yielding pTKY763. This plasmid was then used in a marker exchange experiment in *P. aeruginosa* PAO1.

The $\Delta lasR$ mutant CS9013 was constructed as follows. We generated the plasmid pTKY764 by cloning in pHSG399 a 1,936-bp PCR fragment flanking bp -622 to +1215 relative to the *lasR* translational start site. This plasmid was cleaved at two PstI sites to eliminate the 408-bp central fragment of *lasR*, generating pTKY765. The *lasR*-disrupted fragment in this plasmid was ligated to the vector pEX18. The resultant plasmid, pTKY766, was used in a marker replacement experiment in strain PAO1.

The $\Delta lasI$ mutant CS9038 was constructed as follows. The plasmid pTKY788 was generated by cloning in pHSG399 a 1,080-bp PCR fragment, bp -99 to +981

TABLE 2. Plasmids used in this study

Plasmid	Relevant properties ^a	Reference or source
pTKY613	pUC18 with 1,659-bp fragment containing a part of <i>lon</i>	This study
pTKY614	pTKY613 carrying $\Delta lon::Gm$	This study
pTKY713	pEX18 with HindIII-EcoRI fragment containing disrupted <i>lon</i> gene	This study
pTKY714	pUHE212-1 with 788-bp <i>rhlR</i> fragment	This study
pTKY715	pUHE212-1 with 719-bp <i>rhlI</i> fragment	This study
pTKY762	pTKY714 carrying 574-bp <i>rhlR</i> NruI-NruI region-disrupted gene	This study
pTKY763	pEX18 with EcoRI-HindIII fragment containing disrupted <i>rhlR</i> gene	This study
pTKY764	pHSG399 with 1,936-bp fragment containing <i>lasR</i> gene	This study
pTKY765	pTKY764 carrying 1,528-bp <i>lasR</i> PstI-PstI region-disrupted gene	This study
pTKY766	pEX18 with EcoRI-HindIII fragment containing disrupted <i>lasR</i> gene	This study
pTKY788	pHSG399 with 1,080-bp fragment containing <i>lasI</i> gene	This study
pTKY789	pTKY788 carrying 942-bp <i>lasI</i> BssHII-BssHII region-disrupted gene	This study
pTKY790	pEX18 with BamHI-HindIII fragment containing disrupted <i>lasI</i> gene	This study
pTKY791	pTKY715 carrying 572-bp <i>rhlI</i> PvuII-NruI region-disrupted gene	This study
pTKY792	pEX18 with BamHI-HindIII fragment containing disrupted <i>rhlI</i> gene	This study
pTKY805	pME6032 with MluI-EcoRI fragment carrying the <i>lon</i> region including its own promoter	
pEX18	Gene replacement vector with MCS from pUC18	14
pHSG399	Cloning vector	Our collection
pME6032	Cloning vector	30
pMS255	Gm cassette	2
pUC18	Cloning vector	Our collection
pUHE212-1	N-terminal His tag vector	10

^a Gm, gentamicin resistance.

relative to the *lasI* translational start site. This plasmid was cleaved at two BssHII sites to eliminate the 138-bp central fragment of *lasI*, yielding the plasmid pTKY789. The *lasI*-disrupted fragment in this plasmid was ligated to the vector pEX18. The resultant plasmid, pTKY790, was used in a marker exchange experiment in strain PAO1.

The $\Delta rhlI$ mutant CS9044 was constructed as follows. The plasmid pTKY715 was cleaved at PvuII and NruI sites to eliminate the 147-bp central fragment of *rhlI*, yielding the plasmid pTKY791. The *rhlI*-disrupted fragment in this plasmid was ligated to the vector pEX18. The resultant plasmid, pTKY792, was used in a gene replacement experiment in strain PAO1.

To construct the $\Delta rhlI \Delta lasR$ double mutant CS9051, the plasmid pTKY792 was mobilized into the strain CS9013 ($\Delta lasR$). The transconjugants were selected by resistance to carbenicillin based on a single-crossover event. A double-crossover event in the $\Delta lasR$ mutant was then assessed by its resistance to sucrose and sensitivity to carbenicillin.

To construct the $\Delta lon::Gm$ (CS9008), $\Delta lon::Gm \Delta lasR$ (CS9027), $\Delta lon::Gm \Delta rhlR$ (CS9028), and $\Delta lon::Gm \Delta lasR \Delta rhlI$ (CS9062) mutants, the DNA fragment between nucleotides 814 and 2484 in the *lon* coding region was initially amplified from the chromosome of strain PAO1 by PCR. The 1,659-bp fragment generated was cleaved with HindIII at the 5' end and EcoRI at the 3' end and then cloned into the vector pUC18. The resultant plasmid, pTKY613, was cleaved at the two HincII sites to eliminate the 370-bp central fragment of *lon* and then ligated to the fragment encoding the gentamicin resistance gene generated from SmaI-digested pMS255. The $\Delta lon::Gm$ fragment obtained by digestion of the resultant plasmid, pTKY614, with HindIII and EcoRI was ligated to the vector pEX18. The resultant plasmid, pTKY713, was used to replace $\Delta lon::Gm$ in strains PAO1, CS9011, CS9013, and CS9051.

Generation of the anti-RhlR, anti-RhlI and anti-LasI antisera. To purify the N-terminally His-tagged RhlR and His-tagged RhlI, which had formed inclusion bodies, 3-liter cultures of the *E. coli* DH5 α Z1 derivative harboring pTKY714 or pTKY715 were incubated at 37°C until the cell density reached an optical density at 600 nm (OD_{600}) of 0.8. IPTG (isopropyl- β -D-thiogalactopyranoside) was added to a final concentration of 1 mM for 3 h, and the cells were collected by centrifugation. After one freeze-thaw cycle, the wet cell pastes were resuspended in lysis buffer (100 mM NaH₂PO₄, 10 mM Tris base, 8 M urea, pH 8.0) and sonicated. After centrifugation, the pellets were washed twice with 1% Triton X-100 and once with 0.1% sodium dodecyl sulfate (SDS). After further centrifugation, the precipitates were suspended in SDS sample buffer, following by SDS-polyacrylamide gel electrophoresis (PAGE). The eluates from the gel corresponding to each protein band were used to immunize a rabbit. To generate anti-LasI antiserum, the purified LasI Δ G protein (12) (provided by M. Churchill) was used to immunize a rabbit. The resultant antisera were verified by reaction with the purified antigen proteins.

Comparative analysis of C₄-HSL. The detection of C₄-HSL was based on the method of Shaw et al. (37). Bacterial cells were grown in L broth to stationary phase at 37°C and removed by centrifugation. Samples (80 ml) of the supernatants were filtered through a Millex-GV filter (Millipore) and extracted twice with 50 ml of ethyl acetate. HSL developed on the TLC plate was detected using an indicator bacterium, *Chromobacterium violaceum* mutant CV026, by the method of Latifi et al. (23).

SDS-PAGE and immunoblotting. Gel electrophoresis was carried out according to the method of Laemmli (21) using 15% SDS-polyacrylamide gels. The separated proteins were transferred onto Immobilon-PVDF membranes (Bio-Rad) and then incubated with anti-*E. coli* Lon serum (1:12,500), anti-*Pseudomonas* LasI and anti-RhlR sera (1:25,000), or anti-*Pseudomonas* RhlR serum (1:25,000), followed by alkaline phosphatase-conjugated anti-rabbit immunoglobulin G. The enzymatic reactions were performed in the presence of 0.3 mg ml⁻¹ lysozyme nitroblue tetrazolium (Wako) and 0.15 mg ml⁻¹ 5-bromo-4-chloro-3-indolylphosphate (Sigma).

Pulse-labeling and coimmunoprecipitation. Bacterial cells of strains PAO1 and CS9008 were grown in L broth at 37°C until the cell density reached an OD_{600} of 0.5. The cells were collected by centrifugation and were resuspended in M9 medium supplemented with 0.25% glucose, 40 μ g ml⁻¹ (each) of 18 amino acids (methionine and cysteine were excluded), and 2 μ g ml⁻¹ thiamine. They were then incubated at 37°C for 30 min, labeled with 3.7 Mbq ml⁻¹ [³⁵S]Met and [³⁵S]Cys (Protein Labeling Mix; >37 Tbq mmol⁻¹; GE Health Science) for 1 min, and chased with 200 μ g ml⁻¹ unlabeled methionine and cysteine. Aliquots of the cells were taken at appropriate intervals and mixed with trichloroacetic acid (final concentration, 5%), chilled on ice for 15 min, and centrifuged at 16,000 $\times$ g for 2 min. The pellets were washed with acetone and resuspended in 100 μ l of SDS buffer (10% SDS, 1 mM EDTA, 50 mM Tris-HCl, pH 8.0). One milliliter of RIPA buffer (1% Triton X-100, 1% Na-deoxycholate, 0.1% SDS, 0.15 M NaCl, 50 mM Tris-HCl, pH 8.0, 0.1 mM EDTA) was added to each

resuspended pellet, and the mixture was centrifuged. The supernatants were used for immunoprecipitation with anti-LasI and anti-RhlR sera by incubation overnight on ice. To collect the immunocomplexes, protein A-Sepharose beads (GE Health Science) were added, and the mixtures were incubated for 1 h at 4°C. After centrifugation, the pellets were washed twice with RIPA buffer and once with 10 mM Tris-HCl, pH 8.0, and finally dissolved in 40 μ l of SDS sample buffer (21). A portion of each sample was analyzed by 15% SDS-PAGE. The radioactivity incorporated into LasI was visualized using Molecular Imager FX (Bio-Rad).

RNA extraction and quantitative, real-time reverse transcription (RT)-PCR. Bacterial cells were grown in L broth at 37°C to mid-exponential phase (OD_{600} , 1.0 to 1.4) and for 24 h. A 0.4-ml aliquot of these cultures was added to 0.8 ml of RNeasy Protect Bacterial reagent (Qiagen). Total RNA was isolated by using an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Residual DNA was removed from the RNeasy-Free DNase Set (Qiagen).

Differential expression of genes was examined by quantitative, real-time RT-PCR using a QuantiTect Probe RT-PCR kit (Qiagen) according to the manufacturer's instructions with TaqMan probes and primer pairs designed with the Primer Express software package (ABI). The following probe and primer sequences were used: for *lasI*, sense primer, 5'-GCCCTACATGCTGAAGAACA-3', antisense primer, 5'-CGAGCAAGGCGCTTCCT-3', and probe 5'-(6-carboxyfluorescein [FAM])CTTCCCGAGCTTCTGCACGGC (6-carboxytetramethylrhodamine [TAMRA])-3'; for *rhlI*, sense primer, 5'-G CAGCTGGCGATGAAGATATTC-3', antisense primer, 5'-CGAACGAAA TAGCGCTCCAT-3', and probe, 5'-(FAM)AGCTGCAATGCGCTGGT ACCT(TAMRA)-3'; for *rhlR*, sense primer, 5'-AACGCGAGATCCTGCAA TG-3', antisense primer, 5'-GCGCGTCGAACCTTCTCTG-3', and probe 5'-(FAM)TGAGCATCTCCGAGAGCACGGT(TAMRA)-3'; and for *rplU*, sense primer, 5'-TCACCGAAGGCGAATTCT-3', antisense primer, 5'-TT CACGTCTTCGCCATTGG-3', and probe, 5'-(VIC)ATTTCGACCGCGTC CTGCTGGTT(TAMRA)-3'. To check for residual contaminating genomic DNA, control reactions without the reverse transcriptase mixture were analyzed in the same way. Prior to comparative analysis, the relative efficiency of each probe and primer pair was tested and compared with that of the probe and primer pair for ribosomal *rplU* (referred to as the normalizer gene) to ensure that the threshold cycle (C_T) data analysis approach could be employed. The absolute value of the slope of the log input amount versus ΔC_T was less than 0.1 for all comparisons, which allowed us to use the $\Delta\Delta C_T$ calculation to determine the relative levels of gene expression in all experimental cultures compared to the levels in controls. All reactions were performed in duplicate with an Mx3000P QPCR system (Stratagene), and the experiments were replicated at least three times.

RESULTS

Construction of a *lon*-disrupted mutant of *P. aeruginosa* PAO1. DNA sequence analysis of the *lon* region of *P. aeruginosa* strain PAO1 (GenBank accession number NC_002516) showed that a *lon* homologue encodes a protein with 69.6% identity to the Lon protein of *E. coli*. The open reading frame for Lon is 2,397 bp long and encodes a peptide of 798 amino acid residues with a predicted molecular mass of 88.6 kDa, which is larger than *E. coli* Lon (784 amino acids; 87.4 kDa). The *E. coli* Lon subunit has been shown to comprise three functional domains (35): the N-terminal domain (N; also referred to as LAN), which interacts selectively with target proteins (16); the central ATPase (the AAA⁺ module, or A domain); and the C-terminal proteolytic (P) domain. The deduced amino acid sequence of *P. aeruginosa* Lon shows potential consensus regions in the A and P domains.

A genetically defined *lon* mutant was constructed by inserting a gentamicin resistance gene into *lon*. The disruption in the resultant mutant strain, CS9008, was confirmed by immunoblotting; an antiserum specific for the *E. coli* Lon protein failed to react with a cell lysate from the mutant strain, whereas a band corresponding to approximately 89 kDa was detected in a cell lysate from the wild-type strain (data not shown). Dis-

FIG. 1. Effect of *lon* disruption on C₄-HSL production by *P. aeruginosa*. The extracts from the supernatants of cultures of bacterial strains PAO1 (*lon*⁺) and CS9008 (Δ *lon*) were applied to C₁₈ reversed-phase thin-layer plates and then developed with methanol/water (60:40 [vol/vol]). The spots were visualized with the *C. violaceum* reporter strain. C₄-HSL (0.125 μ mol) and C₆-HSL (0.0125 μ mol) were applied as HSL standards.

ruption of the *lon* gene did not affect the increase in OD used to monitor bacterial growth (data not shown). However, the numbers of viable cells were always lower for the *lon*-disrupted mutant than for the wild-type cells. The *lon*-disrupted mutant exhibited a long-filament phenotype. Because filamentous cells have larger volumes than normal cells, the *lon*-disrupted cells gave ODs similar to those of the wild-type cells despite their lower viability.

During further phenotypic characterization of the *lon*-disrupted mutant, we found that the mutant overproduced a blue-green phenazine pigment, pyocyanin. At 24 h of incubation, the level of pyocyanin in the *lon*-disrupted cells was 4.4-fold higher than in the wild-type cells (data not shown). Pyocyanin is a synthesized by-product of the *phzA1-phzG1* operon, expression of which is under the control of the RhIR/RhII circuit of the QS system. Many genes are regulated by the RhIR/RhII system. One of them, *rpoS*, was also more highly expressed in the *lon*-disrupted cells than in the wild-type cells (data not shown). These findings suggest that Lon protease may be involved in the expression of the RhIR/RhII circuit.

Effect of *lon* disruption on the RhIR/RhII system. To determine whether Lon protease is involved in regulating the RhIR/RhII system, we initially compared C₄-HSL production directed by RhII in the wild-type and *lon*-disrupted mutant strains. The lactones were extracted with ethyl acetate and detected by a bioassay that depended on the induction of violacein in the *C. violaceum* mutant CV026 (47). Besides C₄-HSL, *P. aeruginosa* produces C₆-HSL, which is also synthesized by RhII and is involved in the induction of violacein in CV026 (47). These molecules were separated by C₁₈ reversed-phase thin-layer chromatography. Whereas, only a trace of C₄-HSL was detected in the wild-type strain, the amount of C₄-HSL was dramatically increased in the *lon*-disrupted mutant (Fig. 1); also, more C₆-HSL was detected.

To determine the levels of RhII in both the wild-type and *lon*-disrupted mutant cells, we initially constructed a plasmid encoding His-tagged RhII, isolated the His-tagged protein, and used it to immunize rabbits as described in Materials and Methods. The anti-RhII serum was verified by reaction with

FIG. 2. Expression of *rhII* (A) and *rhIR* (B) in wild-type and *lon*-disrupted cells. Total RNAs were prepared from strains PAO1 (*lon*⁺), CS9008 (Δ *lon*), and CS9053 (Δ *lon plon*⁺) grown in L broth for 24 h 37°C. The levels of *rhII* and *rhIR* transcripts were measured by quantitative, real-time RT-PCR and then normalized to *rplU* expression. The values represent the means and standard deviations of changes in comparison with the transcription level in PAO1.

the purified RhII proteins, but it could not detect the cellular level of RhII in either the wild-type or the *lon*-disrupted cells, suggesting that the protein is short-lived and/or present in small amounts. Therefore, we decided to test the effect of the *lon* disruption on the level of the *rhII* transcript by quantitative RT-PCR (Fig. 2A). The level of *rhII* mRNA was approximately 2.5-fold higher in the *lon*-disrupted cells than in the *lon*⁺ cells. The increase in expression of the *rhII* gene by *lon* disruption was fully suppressed by providing a functional copy of *lon* in *trans*, suggesting that Lon is involved in the expression of *rhII*. The effect of *lon* disruption on the expression of *rhIR* was also examined by measuring the *rhIR* transcript level (Fig. 2B). The *lon* disruption increased *rhIR* transcription fourfold, and this increase was fully suppressed by *lon*. These results suggest that Lon is involved in the expression of both *rhII* and *rhIR*.

Using the anti-RhIR antiserum established in the present study, the levels of RhIR were determined in both wild-type and *lon*-disrupted cells (Fig. 3A). The apparent levels of RhIR are consistent with the transcriptional-analysis results. That is, there was more RhIR in *lon*-disrupted cells than that in *lon*⁺ cells, and the accumulation of RhIR in the *lon*-disrupted cells was suppressed by transcomplementation with functional *lon*. A previous report demonstrated that *E. coli* Clp and Lon proteases are responsible for degrading apo-TraR, a transcriptional activator of the QS system in *Agrobacterium tumefaciens* (48). RhIR and TraR belong to the LuxR-like protein family. Therefore, we examined whether Lon protease degrades RhIR by determining the in vivo half-life of RhIR protein by pulse-labeling and chasing, followed by immunoprecipitation using the anti-RhIR antiserum. As shown in Fig. 3B and C, there was no significant difference between the half-lives of RhIR proteins in wild-type and *lon*-disrupted cells. This excluded the possibility that Lon may degrade the RhIR protein.

Lon regulates the RhIR/RhII system by a LasR/LasI-dependent pathway. Regulation of the RhIR/RhII system is intimately and hierarchically connected to the LasR/LasI system.

FIG. 3. Cellular levels and in vivo stabilities of RhlR. (A) Whole-cell extracts were prepared from strains PAO1 (lon^+ $rhlR^+$), CS9008 (Δlon $rhlR^+$), CS9011 (lon^+ $\Delta rhlR$), and CS9053 (Δlon $plon^+$) grown in L broth for 24 h at 37°C and then separated on 15% SDS-polyacrylamide gels. The separated proteins were transferred to polyvinylidene difluoride membranes and then immunostained with anti-RhlR serum. (B) Cells of strains PAO1 (lon^+) and CS9008 (Δlon) were grown to exponential phase. They were pulse-labeled with [35 S]methionine and [35 S]cysteine for 1 min at 37°C and chased with unlabeled methionine and cysteine. Samples were taken at the times indicated, followed by immunoprecipitation of RhlR. (C) Quantification of the precipitated RhlR protein relative to the value at 1 min. Mean values and standard deviations of at least three independent experiments are given.

The RhlR/RhlI system is subordinate to the LasR/LasI system, since expression of *rhlR* depends on the latter. Two key regulator proteins are involved in activating the LasR/LasI system: the transcriptional activator LasR and the autoinducer synthesis protein LasI. Expression of the hemolysin gene is known to be under the control of the LasR/LasI system. Since hemolytic activity was greater in *lon*-disrupted than in wild-type cells (data not shown), it was suggested that Lon might be involved in modulating the LasR/LasI system, decreasing the cellular level of RhlR. To examine this possibility, we determined the level of LasI protein in bacterial cells. To generate the anti-LasI antiserum, the purified LasI Δ G protein provided by M. Churchill (12) was injected into a rabbit. The immunoblotting result is shown in Fig. 4A. Whereas a little LasI was present in the wild-type cells (lon^+ $lasR^+$), a large amount was detected in the *lon*-disrupted cells (Δlon $lasR^+$), suggesting that Lon modulates the cellular level of LasI. The accumulation of LasI in the *lon*-disrupted cells was completely suppressed by pro-

FIG. 4. Cellular levels of LasI (A) and relative levels of *lasI* expression (B). (A) Whole-cell extracts were prepared from strains PAO1 (lon^+ $lasR^+$), CS9008 (Δlon $lasR^+$), CS9013 (lon^+ $\Delta lasR$), CS9027 (Δlon $\Delta lasR$), and CS9038 (lon^+ $\Delta lasI$) grown in L broth for 24 h at 37°C and then separated on 15% SDS-polyacrylamide gels. The separated proteins were transferred to polyvinylidene difluoride membranes and then immunostained with anti-LasI serum. (B) Total RNAs were prepared from strains PAO1 (lon^+ $lasR^+$), CS9008 (Δlon $lasR^+$), CS9013 (lon^+ $\Delta lasR$), and CS9027 (Δlon $\Delta lasR$) grown in L broth to exponential phase at 37°C. The levels of *lasI* transcript were measured by quantitative, real-time RT-PCR and then normalized to *rplU* expression. The values represent the means and standard deviations of changes in comparison with the transcription level in PAO1.

viding a functional *lon* gene in *trans* (data not shown). Therefore, it is possible that Lon modulates the LasR/LasI system.

To explain the involvement of Lon in regulating the LasR/LasI system, we examined the cellular level of LasI in a *lasR*-disrupted background (Fig. 4A) because the expression of *lasI* is positively regulated by the LasR/3-oxo- C_{12} -HSL complex. The increase in the cellular level of LasI effected by *lon* disruption persisted when *lasR* was additionally disrupted, suggesting that Lon controls the cellular level of LasI protein by a mechanism independent of LasR. The effect of *lon* disruption on *lasI* expression was examined in cells with the mutation background used for immunoblotting analysis (Fig. 4A) by quantitative RT-PCR. The results shown in Fig. 4B demonstrate that *lon* disruption did not affect *lasI* transcription in either $lasR^+$ or $\Delta lasR$ cells. These results suggest that Lon may directly control the level of LasI by degrading it. To test whether Lon is involved in the turnover of LasI, we determined the in vivo half-life of this protein by pulse-labeling and chasing, followed by immunoprecipitation of LasI (Fig. 5A and B). Whereas LasI decayed with a half-life of 6.6 min in the wild-type cells, it did not seem to decay in the *lon*-disrupted cells even after a 20-min chase. The result indicates that Lon is involved in the turnover of LasI protein.

It is well known that *rhlR* expression depends mainly on the LasR/3-oxo- C_{12} -HSL complex during growth in LB broth (31).

FIG. 5. In vivo stabilities of LasI. (A) Cells of strains PAO1 (*lon*⁺) and CS9008 (*Δlon*) were grown to exponential phase and pulse-labeled with [³⁵S]methionine and [³⁵S]cysteine for 1 min at 37°C and then chased with unlabeled methionine and cysteine. Samples were taken at the times indicated, followed by immunoprecipitation of LasI. (B) Quantification of the precipitated LasI protein relative to the value at 1 min. Mean values and standard deviations of at least three independent experiments are given.

To elucidate the role of the LasR/LasI system in the regulation of RhlR/RhlI by Lon, we measured the levels of RhlR in *lon*-disrupted cells after introducing a *lasR* disruption mutation (Fig. 6A). The results showed that the *lasR* mutation decreased the cellular level of RhlR even in the *lon*-disrupted background. Quantitative RT-PCR analysis also demonstrated that the *lasR*-disrupted mutation decreased the level of *rhlR* transcripts (Fig. 6B), suggesting that the LasR/LasI system is involved in regulation of the RhlR/RhlI system by Lon. Taking these findings together, it is suggested that the RhlR/RhlI system is modulated by Lon protease through the LasR/LasI system, where the turnover of LasI is under the control of the Lon protease.

LasR/LasI-independent regulation of the RhlR/RhlI system by Lon. If the RhlR/RhlI system is down-regulated by Lon only through modulation of the LasR/LasI system, the increase in the cellular levels of RhlR due to stabilization of LasI in the *lon*-deficient mutant cells (Fig. 5) would be lost if *lasR* were disrupted. As shown by immunoblotting (Fig. 6A), there was a significant increase in the cellular level of RhlR in the *lon*-disrupted cells (*Δlon ΔlasR*), even in the absence of *lasR*, compared to *lon*⁺ cells (*lon*⁺ *ΔlasR*). Quantitative RT-PCR analysis of *rhlR* transcripts showed that the enhancement of *rhlR* transcription by *lon* disruption was not abolished by introducing the *lasR*-disrupted mutation (Fig. 6B). These results suggest that Lon may regulate expression of the RhlR/RhlI system

FIG. 6. Cellular levels of RhlR (A) and relative levels of *rhlR* expression (B) in the absence of a *lasR* and/or *rhlI* gene in cells. (A) Whole-cell extracts were prepared from strains PAO1 (*lon*⁺ *lasR*⁺ *rhlI*⁺), CS9008 (*Δlon* *lasR*⁺ *rhlI*⁺), CS9013 (*lon*⁺ *ΔlasR* *rhlI*⁺), CS9027 (*Δlon* *ΔlasR* *rhlI*⁺), CS9051 (*lon*⁺ *ΔlasR* *ΔrhlI*), and CS9062 (*Δlon* *ΔlasR* *ΔrhlI*) grown in L broth for 24 h at 37°C and then separated on 15% SDS-polyacrylamide gel. The separated proteins were transferred to polyvinylidene difluoride membranes and then immunostained with anti-RhlR serum. (B) Total RNAs were prepared from strains PAO1 (*lon*⁺ *lasR*⁺ *rhlI*⁺), CS9008 (*Δlon* *lasR*⁺ *rhlI*⁺), CS9013 (*lon*⁺ *ΔlasR* *rhlI*⁺), CS9027 (*Δlon* *ΔlasR* *rhlI*⁺), CS9051 (*lon*⁺ *ΔlasR* *ΔrhlI*), and CS9062 (*Δlon* *ΔlasR* *ΔrhlI*) grown in L broth to exponential phase at 37°C. The levels of *lasI* transcript were measured by quantitative real-time RT-PCR and then normalized to *rplU* expression. The values represent the means and standard deviations of changes in comparison with the transcription level in PAO1.

via a LasR/LasI-independent pathway, in addition to the LasR/LasI-dependent pathway.

We then looked for the LasR/LasI-independent pathway for controlling *rhlR* expression. Since the expression of *rhlI* is known to be autoregulated by the RhlR/RhlI system (13), it is possible that the accumulation of RhlR protein in *lon*-disrupted cells may depend on the RhlI protein. To evaluate this suggestion, we analyzed the effect of *lon* disruption on the cellular level of RhlR in both *lasR*- and *rhlI*-disrupted backgrounds. The result in Fig. 6A shows that the increase in the RhlR level caused by *lon* disruption was suppressed by additional disruption of *rhlI*. A double mutation in *rhlI* and *lasR* simultaneously abolished the increase of *rhlR* transcription by *lon* disruption (Fig. 6B). These results suggest that Lon also regulates the expression of *rhlR* by modulating RhlI, independently of the LasR/LasI system.

DISCUSSION

In the present study, we have demonstrated that Lon protease in *P. aeruginosa* is involved in the regulation of two

HSL-mediated QS systems, LasR/LasI and RhlR/RhlI. First, we showed that LasI accumulated dramatically in *lon*-disrupted cells independently of LasR, which is involved in the autoregulation of *lasI* expression (Fig. 4). Then, we found that LasI was significantly stabilized in *lon*-disrupted cells by determining its half-life in vivo (Fig. 5). We therefore proposed that Lon negatively controls the LasR/LasI system through degradation of LasI, which directs the synthesis of 3-oxo-C₁₂-HSL. The LasR/3-oxo-C₁₂-HSL complex positively regulates *lasI* expression, creating a positive regulatory feedback loop (43). It was therefore assumed that an elevated level of LasI could lead to increased *lasI* transcription. However, this was not actually the case, as indicated in Fig. 4B. It is also known that the complex activates the expression of *rsaL* (between *lasR* and *lasI*), the product of which negatively regulates *lasI* expression (34). Therefore, it is assumed that the accumulation of LasI caused by *lon* disruption could further activate the positive regulator, LasR, and increase the negative regulator, RsaL. This inverse regulation of *lasI* expression by LasR and RsaL could explain why *lon* disruption did not affect *lasI* transcription (Fig. 4) in spite of the accumulation of LasI. QS systems are organized hierarchically: RhlR/RhlI is subordinate to LasR/LasI (9). The present results suggest that Lon is involved in negative regulation of RhlR/RhlI expression through the degradation of LasI. On the other hand, the enhancement of the cellular level of RhlR by *lon* disruption was not completely abolished by the additional disruption of *lasR*. Interestingly, it was abolished by additional *rhlI* disruption (Fig. 6). These results suggest that an additional pathway, independent of the LasR/LasI system but dependent on RhlI, is involved in the regulation of *rhlR* expression by Lon. Taking these findings together, it is suggested that degradation of at least two positive regulators of QS by Lon protease leads to tight regulation of the expression of the two QS systems in *P. aeruginosa*.

A previous report demonstrated that the expression of *rhlR* is mainly induced by the LasR/3-oxo-C₁₂-HSL complex (31). However, the results in Fig. 6 show that the *rhlR* transcript and RhlR accumulate sufficiently even in *lasR*-disrupted cells after *lon* disruption. *rhlR* contains at least four transcription start sites, P1 to P4 (28). Expression from P1 and P4 is dependent on the LasR/3-oxo-C₁₂-HSL complex, and that from P2 and P3 depends on other factors (28). It has been demonstrated that expression of *rhlR* starts from P2 in stationary-phase *P. aeruginosa* PAO1 in LB medium (28). For this reason, sufficient *rhlR* transcripts are produced to lead to the accumulation of RhlR protein in the *lasR*-disrupted background. The result in Fig. 6 also shows that the enhancement of *rhlR* expression by *lon* disruption is abolished by introducing an *rhlI* mutation, suggesting that RhlI possibly activates *rhlR* expression. The pathway through which RhlI is involved in the activation of *rhlR* transcription has not been elucidated. The previous transcriptome analysis demonstrated that the level of *rhlR* transcription activated by both C₄-HSL and 3-oxo-C₁₂-HSL was higher than that activated by 3-oxo-C₁₂-HSL alone (36). Therefore, it can be assumed that the excess levels of C₄-HSL synthesized by RhlI due to *lon* disruption could activate the transcription of *rhlR*.

HSL levels are crucial for controlling QS systems and are therefore subject to regulation by a number of additional mechanisms. For example, the expression of HSL synthase

genes is affected by various regulatory factors. The transcription of *lasI* is activated by the LasR/3-oxo-C₁₂-HSL complex, VqsR (20), and PprB (7) and is repressed by RsaL (6). The LasR/3-oxo-C₁₂-HSL complex and PprB (7) regulate *rhlI* transcription positively, but RpoS (46), RpoN (40), and Dks (19) regulate it negatively. RsaA could be involved in the stability of *lasI* mRNA (33). In addition to transcriptional control, our present study indicates that posttranslational control of LasI is important in regulating the HSL level, leading to negative control of QS system expression in *P. aeruginosa*.

Very recently, Bertani et al. reported that *P. putida* Lon protease is involved in regulating the HSL-mediated QS system PpuR/PpuI (3). They assumed that Lon was involved in regulating *ppuI* expression via the degradation of a LuxR-type protein, PpuR, on the basis of a previous report in which Lon and another ATP-dependent protease, ClpXP, were shown to be involved in degrading TraR, a LuxR-type protein of *A. tumefaciens*, in the absence of HSL (48). However, Lon could not degrade TraR in the presence of HSL. In our results, *lon* disruption did not affect the stability of the LuxR-type protein RhlR in the presence (Fig. 3) or absence (data not shown) of HSL. Furthermore, another LuxR-type protein from *Erwinia carotovora* was shown to be more sensitive to trypsin in vitro in the absence of HSL (45), suggesting that this protein might be nonspecifically degraded by proteases. Since *ppuI* expression is involved in the positive-induction loop of the PpuR/PpuI system, Lon in *P. putida* might possibly regulate *ppuI* expression by degrading PpuI. All HSL-mediated QS systems in many gram-negative bacteria are constructed by LuxR/I-type proteins. Since Lon protease is conserved, it might be generally involved in regulating all HSL-mediated QS systems.

ACKNOWLEDGMENTS

We thank T. Tomoyasu for helpful discussions. We also thank T. Nemoto for technical assistance. We are grateful to M. E. A. Churchill for supplying the LasIΔG protein, to N. Gotoh for supplying the plasmid pME6032, to Y. Tsuda for supplying plasmids pEX18 and pMS255, and to T. Ikeda for supplying the *C. violaceum* strain CV026 and C₄-HSL.

This research was supported in part by grants-in-aid for scientific research 16790252 and 19790313 to A.T. from the Ministry of Education, Culture, Sports, Science and Technology of the Japanese government.

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