Lon Protease Influences Antibiotic Production and UV Tolerance of *Pseudomonas fluorescens* Pf-5

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Pseudomonas fluorescens Pf-5 is a soil bacterium that suppresses plant pathogens due in part to its production of the antibiotic pyoluteorin. Previous characterization of Pf-5 revealed three global regulators, including the stationary-phase sigma factor σ^S and the two-component regulators GacA and GacS, that influence both antibiotic production and stress response. In this report, we describe the serine protease Lon as a fourth global regulator influencing these phenotypes in Pf-5. *lon* mutants overproduced pyoluteorin, transcribed pyoluteorin biosynthesis genes at enhanced levels, and were more sensitive to UV exposure than Pf-5. The *lon* gene was preceded by sequences that resembled promoters recognized by the heat shock sigma factor σ^{32} (σ^H) of *Escherichia coli*, and Lon accumulation by Pf-5 increased after heat shock. Therefore, σ^H represents the third sigma factor (with σ^S and σ^{70}) implicated in the regulation of antibiotic production by *P. fluorescens*. Lon protein levels were similar in stationary-phase and exponentially growing cultures of Pf-5 and were not positively affected by the global regulator σ^S or GacS. The association of antibiotic production and stress response has practical implications for the success of disease suppression in the soil environment, where biological control organisms such as Pf-5 are likely to encounter environmental stresses.

Pseudomonas fluorescens is a ubiquitous soil microorganism that inhabits the surfaces of seeds and roots. Some strains of *P. fluorescens*, when growing in association with plants, can protect them from infection by plant pathogens (50). One such strain, *P. fluorescens* Pf-5, produces a number of antibiotics, including pyoluteorin (Plt) (21), pyrrolnitrin (Prn) (20), and 2,4-diacetylphloroglucinol (Phl) (39). Of the three antibiotics, Plt is most toxic to the oomycete *Pythium ultimum* (34), which can infect seeds and roots of many plant hosts and cause seedling death and root rot (33).

Antibiotic production by *P. fluorescens* is controlled by several global regulatory genes that influence multiple phenotypes, including stress tolerance, and also by regulatory genes linked to antibiotic biosynthesis gene clusters. For example, Plt production by Pf-5 is controlled by *pltR*, a member of the LysR family of transcriptional activators that is linked to the Plt biosynthesis genes *pltLABCDEFG* and *pltM* (38), and the global regulatory genes *gacA*, *gacS*, and *rpoS*. GacA and GacS constitute a two-component regulatory system that is required for the production of antibiotics, exoenzymes, and virulence factors by many *Pseudomonas* spp. (8, 25). Derivatives of Pf-5 harboring mutations in *gacA* and *gacS* do not produce Plt, Prn, or Phl (8, 53) and are impaired in their tolerance to oxidative stress (53). The stationary-phase sigma factor σ^S, encoded by *rpoS*, has a differential effect on antibiotic production by Pf-5; an *rpoS*::Tn5 mutant does not produce Prn but overproduces Plt and Phl and is superior to Pf-5 in suppressing Pythium damping-off of cucumber (46). The *rpoS*::Tn5 mutant is also impaired in its tolerance to oxidative and osmotic stress (46). GacA and GacS are necessary for the timely expression and accumulation of σ^S during the transition between exponential growth and stationary phase, indicating that GacA and GacS influence a regulatory circuit in which σ^S is a participant (53).

Characterization of *gacA*, *gacS*, and *rpoS* in Pf-5 has provided a glimpse into the intricate regulatory networks controlling antibiotic production in *P. fluorescens*. In this study, we cloned and sequenced a fourth global regulatory gene influencing antibiotic production by Pf-5. We identified the gene as a homolog of *lon*, which encodes an ATP-dependent serine protease (17, 18) found in diverse organisms, including bacteria, plants, and animals. In *Escherichia coli*, Lon is a heat shock protein that nonspecifically degrades denatured or nonfunctional intracellular proteins (17, 18). Lon also functions in gene regulation by specifically degrading unstable regulatory proteins (17, 18). In *Bacillus subtilis*, *lon* expression is induced by salt and oxidative stress (43) as well as starvation (19). Because resistance to salt, oxidation, and starvation is regulated by σ^S in Pf-5 (46; V. O. Stockwell, unpublished data) and *lon*::Tn5 and *rpoS*::Tn5 mutants are similar in their overproduction of Plt (27, 46), we evaluated the influence of σ^S on Lon accumulation. Lon protein levels did not increase during stationary phase and were not reduced in *rpoS* mutants of Pf-5, as would be expected for proteins positively regulated by *rpoS*. We also evaluated two stress responses that are influenced by Lon in other bacterial genera. Lon of Pf-5 was required for optimal tolerance of Pf-5 to UV irradiation, and Lon protein was induced by heat shock.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used are listed in Table 1. *P. fluorescens* was grown at 27°C with shaking at 200 rpm in King's medium B (KB) broth (24) for routine culturing; in nutrient broth (NB) (Difco Laboratories, Detroit, Mich.) supplemented with 2% (wt/vol) glucose or 1% (wt/vol) glycerol for antibiotic extractions; in NB supplemented with 1% (wt/vol) glycerol for ice nucleation assays; in M9 minimal medium (M9) supplemented with 0.4% glucose (45) for Western analysis; or in Luria-Bertani medium (LB) (45) for UV stress tests and Western analysis. Cultures of *E. coli* were grown in LB at 37°C. For cultures of *E. coli*, antibiotic concentrations were 100 µg of ampicillin (Ap) per ml, 12 µg of gentamicin (Gm) per ml, 50 µg of kanamycin (Km) per ml, and 20 µg of tetracycline (Tc) per ml.

Recombinant DNA techniques. Genomic DNA was isolated by cetyltrimethylamonium bromide with isopropanol precipitation (3). Plasmids were purified by an alkaline lysis procedure (45). Methods for transformations, digestions with restriction enzymes, and gel electrophoresis were standard (45). Enzymes were

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference(s)
P. fluorescens:		
Pf-5	Rhizosphere isolate	20
JL3985	*rpoS*::Tn5 derivative of Pf-5, Km^r	42, 46
JL4292	*lon*::Tn5 derivative of Pf-5, Km^r	27
JL4389	*pltB*::Tn3-nice derivative of Pf-5, Plt$^-$ Ina$^+$ Km^r	28
JL4479	*lon*::Tn5 derivative of Pf-5 obtained by marker exchange mutagenesis, Km^r	This study
JL4490	*rpoS*::*lacZ* derivative of Pf-5	53
JL4590	Δ*hupB*::*aphI* derivatives of Pf-5 obtained by marker exchange mutagenesis, Km^r	This study
JL4591	Δ*hupB*::*aphI* derivatives of Pf-5 obtained by marker exchange mutagenesis, Km^r	This study
JL4594	*lon*::*aacC1* derivative of JL4389 obtained by marker exchange mutagenesis, Ina$^+$ Gm^r Km^r	This study
JL4600	*rpoS*::*lacZ* derivative of JL4389 obtained by marker exchange mutagenesis, Ina$^+$ Lac$^+$ Km^r	This study
JL4601	JL4389 harboring pJEL5649, Ina$^+$ Tc^r	This study
JL4619	*lon*::*aacC1* derivative of Pf-5 obtained by marker exchange mutagenesis, Gm^r Km^r	This study
JL4620	*lon*::*aacC1* *rpoS*::*lacZ* derivative of Pf-5 obtained by marker exchange mutagenesis, Gm^r Km^r	This study
JL4621	*lon*::*aacC1* *rpoS*::*lacZ* derivative of JL4389 obtained by marker exchange mutagenesis, Ina$^+$ Gm^r Km^r	This study
E. coli		
DH5α	F$^-$ *endA1 hsdR17* (r$_k^-$ m$_k^+$) *supE44 thi-1 recA1 gyrA96 relA1* φ80*dlacZ*ΔM15 λ$^-$	45
SG20781	*lon$^+$ cps*::*lac*	6
Plasmids		
pBR322	ColE1 replicon, Ap^r Mob$^+$ Tc^r	45
pRK415	IncP1 replicon, polylinker of pUC19, Mob$^+$ Tc^r	23
pRK2013	Mobilizing plasmid, Tra$^+$ Km^r	14
pLAFR3	IncP1 replicon; *cos* Mob$^+$ Tc^r	29
pMGm	ColE1 replicon, source of *aacC1* (Gm^r) cassette on a 2.0-kb *Sma*I fragment, Gm^r Tc^r	37
pMKm	ColE1 replicon, source of *aphI* (Km^r) cassette on a 1.7-kb blunted *Xho*I fragment, Km^r Tc^r	37
pUC19	ColE1 replicon, Ap^r	45
pUC18	ColE1 replicon, Ap^r	45
pJEL01	Stably maintained in *E. coli* or *Pseudomonas* spp., replicons from pVSP1 and pACYC184, Mob$^+$ Tc^r	46
pJEL5500	2.9-kb *Eco*RI fragment containing *rpoS* from Pf-5 cloned in pUC19, Ap^r	46
pJEL5649	2.9-kb *Eco*RI fragment containing *rpoS* from Pf-5 cloned in pJEL01, Mob$^+$ Tc^r	46
pJEL5913	9.7-kb *Eco*RI genomic fragment from JL4292 containing Tn5 and adjacent DNA cloned into pBR322, Ap^r Tc^r	This study
pJEL5922	3.6-kb *Hind*III-*Eco*RI genomic fragment from pJEL5913 containing 1.1 kb of Tn5 and flanking DNA from JL4292 cloned in pBR322, Ap^r Tc^r	This study
pJEL5926	*rpoS*::*lacZ* transcriptional fusion cloned in pRK415, Mob$^+$ Tc^r	53
pJEL6023	4.3-kb *Hind*III genomic fragment from Pf-5 containing *lon* and *hupB* genes in pUC19, Ap^r	This study
pJEL6161	5.7-kb *Hind*III fragment containing Δ*hupB*::*aphI* cloned in pUC19, Ap^r	This study
pJEL6195	2.3-kb *Eco*RI genomic fragment from Pf-5 containing the upstream regulatory region of *lon* cloned into pUC18, Ap^r	This study
pJEL6197	6.3-kb *Hind*III genomic fragment containing *aacC1* cassette from pMGm inserted into blunted *Sun*I site internal to *lon* in pRK415, Mob$^+$ Gm^r Tc^r	This study
pJEL6206	Cosmid library clone of Pf-5 containing *lon* and *hupB* cloned in pLAFR3, Tc^r	This study

from Gibco-BRL Life Technologies (Gaithersburg, Md.). Ends of restriction fragments were blunted with the large subunit of DNA polymerase (45), and thermal cycling was used for blunt-end ligations (32).

Cloning of *lon* and the linked gene *hupB* from Pf-5. A pLAFR3 genomic library of Pf-5 (42) was screened by colony hybridization (16) to identify cosmids containing wild-type DNA corresponding to the mutagenized locus in the Plt-over-producing mutant JL4292 (Fig. 1a). The probe was a 9.7-kb *Eco*RI fragment containing Tn5 and flanking DNA from the genome of JL4292, which was cloned in pJEL5913 (Fig. 1b). The probe was labeled with digoxigenin-11-dUTP using a nick translation kit (Gibco-BRL Life Technologies). Qiabrane filters (Qiagen, Chatsworth, Calif.) were prepared and hybridized following the methods for the Genius system of Boehringer-Mannheim (Indianapolis, Ind.). Cosmids were isolated from colonies that hybridized to the probe, digested with *Hind*III or *Eco*RI, and analyzed in Southern blots. A 4.3-kb *Hind*III fragment from a cosmid that hybridized to the probe was identified by Southern analysis (data not shown) and cloned into pUC19 to construct pJEL6023 (Fig. 1b). From the same cosmid, an overlapping 2.3-kb *Eco*RI fragment that hybridized to the digoxigenin-11-dUTP-labeled 4.3-kb *Hind*III fragment from pJEL6023 was cloned into pUC18 to create pJEL6195.

Sequence analysis. DNA sequencing and oligonucleotide syntheses were performed at the Center for Gene Research and Biotechnology at Oregon State University in Corvallis. Sequencing of double-stranded templates was performed on an ABI model 373A automated DNA sequencer using a *Taq* DyeDeoxy Terminator Cycle sequencing kit (Applied Biosystems, Inc., Foster City, Calif.) according to the manufacturer's protocol. Oligonucleotide primers were synthesized on an ABI model 380B DNA synthesizer using phosphoramidite chemistry (1). Sequencing of *lon* and *hupB* cloned in pJEL6023 was initiated using oligonucleotide primers complementary to pUC19 DNA on either side of the polylinker and continued using primers complementary to sequenced DNA. Sequencing of the region upstream of *lon* was completed with primers complementary to previously sequenced DNA. The precise location of Tn5 in mutant JL4292 was determined from primers complementary to a terminal region of Tn5

using pJEL5922, a subclone of pJEL5913, as a template. Analyses of DNA and deduced protein sequences and comparisons with sequences in the GenBank database were accomplished with software from the Genetics Computer Group, Inc., Madison, Wis. (10) and the Staden software package (4).

Antibiotic quantification. Antibiotics were extracted from cells and spent medium of cultures grown in triplicate by described methods (46). Plt and Prn concentrations were quantified from cultures grown for 2 days at 20°C in 5 ml of NB containing 1% glycerol, a medium that favors their production. The concentration of Phl was quantified from cultures grown for 4 days in 5 ml of NB containing 2% glucose, a medium that favors its production. After centrifugation of the cultures at $5,000 \times g$ for 5 min, the bacterial pellet was suspended in 4 ml of acetone, and the suspension was sonicated in an ultrasonic cleaner for 30 s. Cell suspensions were centrifuged at $5,000 \times g$ for 10 min, and the acetone supernatant was removed and dried under reduced pressure. Culture supernatants were adjusted to pH 2.0 with 1 M HCl and extracted twice with 2 ml of ethyl acetate. The organic phases were combined and dried under reduced pressure. Extracts dissolved in methanol were analyzed by thin-layer chromatography (TLC) or high-performance liquid chromatography (HPLC). Organic extracts were separated on TLC plates (KC18F; Whatman International Ltd., Maidstone, England) in chloroform-acetone (9:1, vol/vol) and sprayed with diazotized sulfanilic acid for visualization of compounds (44). Antibiotics were detected by their characteristic colors and R_f values, which conformed to those of authentic standards on TLC plates: Plt, $R_f = 0.32$, brown; Prn, $R_f = 0.81$, maroon; and Phl, $R_f = 0.64$, yellow. Antibiotics were separated by HPLC with a Waters Nova-pak radial compression cartridge (0.8 by 10 cm) with an 18-min linear gradient from 10 to 100% acetonitrile with 0.1% acetic acid in water at a flow rate of 1 ml/min (Plt and Prn) or with water-acetonitrile-methanol (45:30:25, vol/vol) at a flow rate of 1.5 ml/min (Phl). Antibiotics were detected with a UV photodiode array detector at 310 nm (Plt), 225 nm (Prn), and 278 nm (Phl) and quantified against authentic standards. Antibiotics, reported as micrograms per milliliter ± standard deviation, were quantified by HPLC from two replicated experiments with similar results, and data from one experiment are presented.
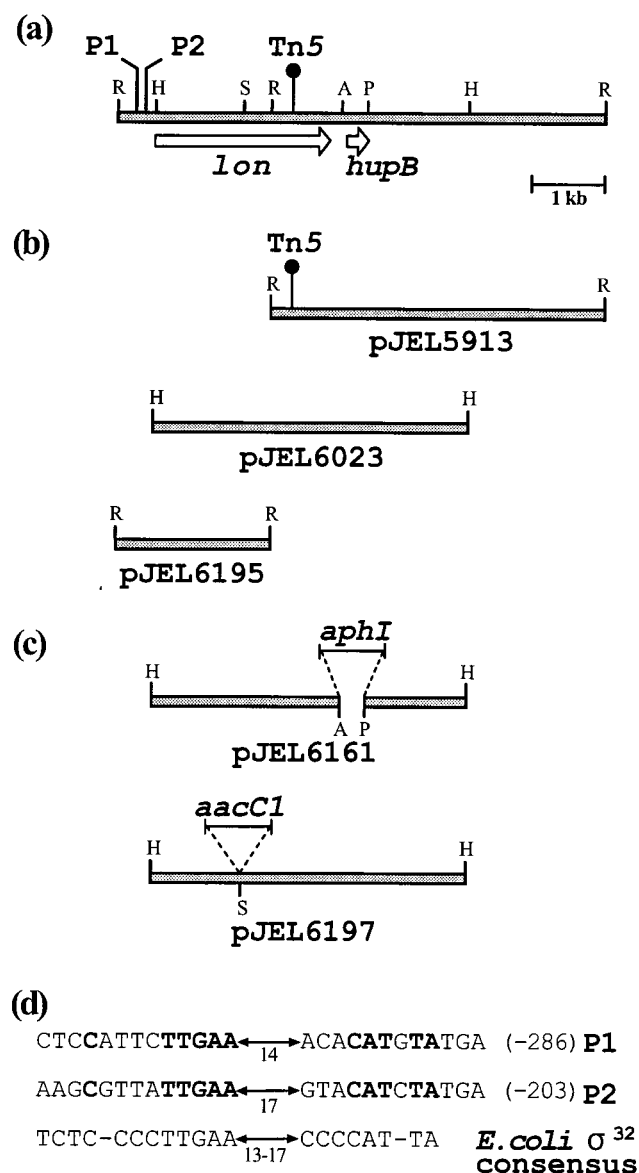
FIG. 1. Schematic representation of the genomic region of Pf-5 containing *lon* and *hupB*. (a) Restriction map of the locus and location of Tn*5* in the *lon* mutants JL4292 and JL4479. Restriction enzymes: A, *Afl*II; H, *Hin*dIII; P, *Hpa*I; R, *Eco*RI; S, *Sun*I. (b) Cloned genomic fragments used in sequence analysis and as probes for Southern blot analysis or for colony hybridization. (c) Schematic representation of cloned genomic fragments with insertions in *lon* and *hupB*. (d) Two potential promoters, P1 at −286 and P2 at −203 relative to the translational start site of *lon* in Pf-5. Nucleotides in bold are identical to the σ^{32} promoter consensus sequence of *E. coli*. Numbers beneath arrows represent nucleotides separating the consensus regions.

Derivation of *lon*, *rpoS*, and *hupB* mutants by marker exchange mutagenesis.
(i) *lon*::Tn*5* mutant. A 9.7-kb Tn*5*-containing *Eco*RI fragment from the genome of JL4292 was cloned into pBR322, which does not replicate in *Pseudomonas* spp., to create pJEL5913 (Fig. 1b). pJEL5913 was mobilized from *E. coli* DH5α donors into Pf-5 in a triparental mating with *E. coli* DH5α containing the helper plasmid pRK2013. Transconjugants from this mating were selected on KB containing streptomycin (100 μg/ml) (to counterselect against *E. coli* donors) and kanamycin (50 μg/ml). The resultant marker-exchanged mutant JL4479 had a Tn*5* insertion in the same region as the original mutant JL4292, as determined by Southern analysis (data not shown) with the 9.7-kb *Eco*RI fragment from pJEL5913 as a probe.

(ii) *lon*::*aacC1* mutants. We evaluated the effect of a mutation in *lon* on *pltB* transcription using existing fusions of the promoterless ice nucleation reporter gene in Tn*3-nice* to the promoter of *pltB* (*pltB*::Tn*3-nice*) (28, 29). Because Tn*5*

and Tn*3-nice* both confer resistance to kanamycin, the 2.0-kb *Sma*I fragment containing *aacC1* from pMGm, which confers gentamicin resistance, was cloned into the blunted *Sun*I site internal to *lon* in pJEL6023 (Fig. 1). The resulting 6.3-kb *Hin*dIII fragment, containing *lon*::*aacC1*, was cloned into pRK415, which confers resistance to tetracycline and is not stably maintained in Pf-5, to create pJEL6197. pJEL6197 was mobilized into Pf-5 as described for the *lon*::Tn*5* mutant, and transconjugants were selected on KB supplemented with tetracycline (200 μg/ml), which selects for pRK415 in Pf-5 and counterselects against *E. coli* donors. To allow loss of the plasmid, Tc^r Gm^r transconjugants were grown in KB broth without antibiotics at 27°C with shaking at 1 to 3 days with daily subculturing. The resultant culture was spread on KB containing gentamicin (40 μg/ml), and individual Gm^r colonies were screened for loss of pJEL6197 by lack of growth on KB containing tetracycline (200 μg/ml). *lon*::*aacC1* was introduced into Pf-5 and JL4389, which contains *pltB*::Tn*3-nice*. In the resultant marker-exchanged mutants JL4619 and JL4594, respectively, *lon*::*aacC1* replaced *lon*, as determined from Southern analysis (data not shown) with the 4.3-kb *Hin*dIII fragment from JL6203 as a probe (Fig. 1b).

(iii) *rpoS*::*lacZ* mutant. Plasmid pJEL5926, which contains an *rpoS*::*lacZ* fusion (53), was used for marker exchange mutagenesis of JL4389, which contains *pltB*::Tn*3-nice*, as described for *lon*::*aacC1*. The resultant marker-exchanged mutant JL4600 was confirmed by Southern analysis (data not shown) with a 2.9-kb *Eco*RI fragment from JL5500 as a probe.

(iv) *rpoS*::*lacZ lon*::*aacC1* double mutants. The *lon*::*aacC1* mutation was exchanged with *lon* in the genome of two *rpoS*::*lacZ* derivatives of Pf-5, strain JL4490 (53) and strain JL4600, which contains *pltB*::Tn*3-nice*, as described above. The resultant marker-exchanged mutants, JL4620 and JL4621, respectively, were confirmed by Southern analysis (data not shown) with the 4.3-kb *Hin*dIII fragment from JL6203 as a probe (Fig. 1b).

(v) Δ*hupB*::*aphI* mutant. The *hupB* gene cloned in pJEL6023 was deleted by digesting plasmid DNA with *Afl*II and *Hpa*I, blunting the ends of the digested DNA, and inserting the 1.7-kb *aphI* cassette from pMKm to derive pJEL6161 (Fig. 1c). The resulting 5.7-kb *Hin*dIII fragment was cloned into pRK415 and used to mutagenize Pf-5 as described for *lon*::*aacC1*. Replacement of *hupB* with Δ*hupB*::*aphI* in two independently derived marker-exchanged mutants, JL4590 and JL4591, was confirmed by Southern analysis (data not shown) with the 4.3-kb *Hin*dIII fragment of pJEL6023 as a probe (Fig. 1b).

Transcription of Plt biosynthetic genes assessed with an ice nucleation reporter gene in Tn*3-nice*. The effect of *rpoS* and *lon* on transcription of the Plt biosynthetic genes was determined by comparing ice nucleation activity expressed by Pf-5 containing genomic insertions of Tn*3-nice* in *pltB* (JL4389) (28) to derivative strains with *rpoS*::*lacZ* (JL4600) (53), with *lon*::*aacC1* (JL4594), with both *rpoS*::*lacZ* and *lon*::*aacC1* mutations (JL4621), or with multiple plasmid-borne copies of *rpoS* (JL4601). Ice nucleation activity was quantified by a droplet-freezing assay at −5°C as described previously (31) from cultures grown for 2 days at 20°C with shaking at 200 rpm in NB amended with 1% glycerol. Cultures were grown in triplicate, the experiment was done twice, and the results of a representative experiment are presented.

Western analysis of Lon and σ^S. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transblotting of total protein extracts for Western analysis were done using the manufacturer's protocols (Bio-Rad Laboratories, Hercules, Calif.) and reported methods (11, 51). Bacterial cultures were grown in LB or M9 supplemented with 0.4% glucose at 27°C with shaking at 200 rpm. For each *P. fluorescens* culture, 30 μg of protein (Bio-Rad DC protein assay) from whole-cell extracts was boiled in sample buffer containing 2-mercaptoethanol and separated on a sodium dodecyl sulfate–8% polyacrylamide gel. A 10-μg amount of protein (Bio-Rad DC protein assay) from whole-cell extracts of *E. coli* was loaded on the gel as a positive control. Protein samples were transferred from the gel onto a nitrocellulose membrane, and the membrane was incubated with antibodies to the Lon protein from *E. coli* (11), which were detected by enhanced chemiluminesence as specified by the manufacturer (Amersham Pharmacia Biotech Inc., Piscataway, N.J.). The amount of Lon in strains of *P. fluorescens* was quantified by using a Molecular Dynamics Personal Densitometer model SI and Image-Quant software V4.1 (Sunnyvale, Calif.) and confirmed to be within the linear range of detection, as described previously (53). The amount of Lon is reported relative to the amount detected in Pf-5 growing exponentially at 27°C. σ^S was quantified from these and similar blots with 10 μg of protein per sample after blots were stripped according to the manufacturer's protocols (Amersham) and reprobed with antibodies to σ^S from *E. coli* (49). Each experiment was replicated with similar results.

Sensitivity to UV irradiation. Cultures were grown with shaking at 27°C in LB, and stationary-phase cells were obtained 4 h after the optical density ($\lambda = 600$ nm) of cultures stopped increasing. Cells were pelleted, washed, serially diluted in 10 mM phosphate buffer (pH 7), and spread onto duplicate or triplicate LB agar plates. Agar plates were exposed to UV irradiation ($\lambda = 254$ nm) at a level of 10 erg/mm^2 for various durations. Colonies arising from surviving cells were counted following 48 h of incubation in the dark. The experiment was done twice with similar results.

Nucleotide sequence accession number. The GenBank accession number for the DNA sequence of the *lon* and *hupB* genes of *P. fluorescens* Pf-5 is AF250140.

TABLE 2. Antibiotic production by *P. fluorescens* Pf-5
and derivatives[a]

Strain	Relevant characteristics	Mean production (µg/ml) ± SD ($n = 3$)	
		Plt	Prn
Pf-5	Wild type	5.3 ± 1.2	1.04 ± 0.26
JL4292	*lon*::Tn*5*	117 ± 11	1.42 ± 0.10
JL4479	*lon*::Tn*5*	101 ± 14	1.44 ± 0.12
JL4619	*lon*::*aacC1*	96 ± 4	1.31 ± 0.13
JL4620	*lon*::*aacC1 rpoS*::*lacZ*	164 ± 13	0.32 ± 0.07
JL4490	*rpoS*::*lacZ*	145 ± 13	0.11 ± 0.02
JL3985	*rpoS*::Tn*5*	149 ± 9	0.38 ± 0.04
JL4590	Δ*hupB*::*aphI*	4.2 ± 0.6	0.83 ± 0.18

[a] Plt and Prn were extracted from cells and spent medium of cultures grown for 2 days at 20°C in 5 ml of NB containing 1% glycerol.

RESULTS

Identification of a *lon*::Tn*5* derivative of Pf-5 that overproduces Plt. JL4292, a derivative of Pf-5 obtained following random Tn*5* mutagenesis, overproduces the antibiotic Plt (27). To confirm that the Tn*5* insertion caused overproduction of Plt by JL4292, the transposon was reintroduced into the same site in the genome of Pf-5 by marker exchange mutagenesis to create JL4479. JL4292 and JL4479 each contained a single Tn*5* insertion in a 9.7-kb *Eco*RI fragment of genomic DNA, as determined from Southern analysis (data not shown) using the Tn*5*-containing *Eco*RI fragment from the genome of JL4292 as a probe (Fig. 1b). Both JL4292 and JL4479 produced a higher concentration of Plt than did Pf-5 (Table 2), confirming that Plt overproduction by JL4292 was associated with the Tn*5* insertion and not due to secondary mutations at other loci. Pf-5, JL4292, and JL4479 did not differ significantly in their production of Phl in two replicate experiments (data not shown).

We began our analysis of the locus disrupted in JL4292 (Fig. 1a) by identifying cosmids containing the corresponding wild-type DNA from an extant genomic library of Pf-5 (42). Cosmids that hybridized to the Tn*5*-containing *Eco*RI fragment from the genome of JL4292 were identified. From one such cosmid, a 4.3-kb *Hin*dIII fragment that hybridized to the probe was cloned (pJEL6023) (Fig. 1b) and used as a template for sequencing. Within the 4.3-kb *Hin*dIII fragment, an open reading frame (ORF) of 2,397 bases was identified, with the putative ribosome-binding site GAGG located 8 bases upstream of an ATG start codon. In JL4292, Tn*5* disrupted the ORF at base 1798 of the coding sequence. Once translated, the ORF was predicted to encode a peptide of 798 amino acids, with 70% identity to Lon of *E. coli* (7). Within the deduced amino acid (a.a.) sequence of Lon from Pf-5, we located all of the characteristic Lon protein domains. From a.a. residues 206 to 266, we located a charged region (a.a. 206 to 221, 53% basic; a.a. 227 to 249, 52% acidic; and a.a. 251 to 266, 31% basic). This region is predicted to form a coiled-coil structure in *E. coli* (13), a common motif involved in protein-protein interactions. Within the acidic portion of the charged region, we located a sequence identical to the discriminator activity consensus (KAIQKELGD from a.a. 232 to 240) from Lon of *E. coli* (13). A mutation within this sequence abolishes activity of Lon towards two specific substrates in *E. coli* (13). ATP-binding motifs (15, 52) that matched the corresponding motifs in Lon of *E. coli* (7) were located from a.a. 352 to 359 (GPPGVGKT) and from a.a. 405 to 420 (KVGVRNPLFLLD). A putative catalytic site serine (2) is at a.a. residue 674. In JL4292, Tn*5* spa-

tially separates the conserved serine residue from the ATP-binding motif and the discriminator activity consensus.

The upstream regulatory region of *lon*, including its promoter, was not located within the 4.3-kb *Hin*dIII fragment cloned in pJEL6023. Therefore, an overlapping *Eco*RI fragment was cloned into pUC18 to create pJEL6195 (Fig. 1b), which was a template for sequence analysis. In the sequence upstream of *lon*, we identified two potential promoters (Fig. 1d), one 203 bases upstream and the other 286 bases upstream of the start codon. The sequences of both promoter regions resembled the consensus sequence recognized by the σ^{32} (σ^H) form of RNA polymerase, which is located upstream of various heat shock-inducible genes in *E. coli*, including *lon* (55).

Δ*hupB*::*aphI* derivative of Pf-5 overproduced Phl but did not overproduce Plt. A second ORF with a predicted GTG start codon was identified 148 bases downstream of the *lon* ORF. The deduced protein encoded by the ORF is 90 amino acids in length and is 80% identical to the histone-like protein HU from *Pseudomonas aeruginosa* (9) encoded by *hupB*. The *hupB* gene in *E. coli* encodes one of two subunits of the heterodimeric protein that is involved in regulating transcription by constraining DNA supercoils and DNA accessibility to regulatory proteins (12). In the genome of *E. coli*, *hupB* is located downstream of *lon*, which further supports our designations for the ORFs as *lon* and *hupB* homologs. Within the intergenic region between *lon* and *hupB*, a putative rho factor-independent terminator-like sequence was identified (5) as a run of consecutive thymine residues. However, a discernible region of dyad symmetry that characterizes terminators did not immediately precede the sequence. Without more convincing evidence of a terminator between *lon* and *hupB*, we chose to test the possibility that a polar effect of *lon*::Tn*5* on *hupB* was responsible for Plt overproduction in JL4292 by deleting *hupB* from Pf-5 and testing the resulting strains for Plt production.

Two Δ*hupB*::*aphI* mutants, JL4590 and JL4591, were generated by marker exchange mutagenesis and confirmed not to overproduce Plt, as assessed by TLC (data not shown). Both JL4590 and JL4591 were more mucoid on KB plates and more viscous in NB culture than Pf-5. Strain JL4590 was further evaluated by HPLC analysis, which revealed that the Δ*hupB*::*aphI* mutant did not differ from Pf-5 in its production of Plt (Table 2). Therefore, the possibility that the Tn*5* insertion into *lon* enhanced Plt production by blocking readthrough transcription of *hupB* was discounted. JL4590 produced more Phl than was produced by Pf-5 in parallel cultures (38.6 ± 8.3 and 13.1 ± 0.7 µg/ml, respectively) grown in NB containing 2% glucose.

Lon protease and σ^S influenced *pltB* biosynthetic gene transcription. The influence of Lon and σ^S on the transcription of *pltB* was assessed with transcriptional fusions of the ice nucleation reporter gene in Tn*3-nice* to *pltB*. Ice nucleation activity expressed by JL4389, which has an insertion of Tn*3-nice* in the genomic *pltB* gene, was compared to the activity of derivative strains with a *lon*::*aacC1* or *rpoS*::*lacZ* mutation, with both the *lon*::*aacC1* and *rpoS*::*lacZ* mutations, or with multiple plasmid-borne copies of *rpoS* (Table 3). Ice nucleation activity is expressed as $\log_{10}$ (ice nuclei per cell); therefore, increasingly positive values represent higher *pltB* transcription, whereas increasingly negative values represent lower *pltB* transcription. In derivatives of JL4389, mutations in *rpoS* and *lon* significantly increased *pltB* transcription compared to strains with functional *rpoS* and *lon*, and transcription in a strain harboring both mutations was further enhanced. Multiple plasmid-borne copies of *rpoS* significantly reduced *pltB* transcription compared to that in strains with a single genomic copy of *rpoS*.

TABLE 3. Influence of *lon* and *rpoS* on transcription of the
Plt biosynthesis gene *pltB*, assessed with an ice
nucleation reporter gene in Tn*3-nice*

Strain	Relevant characteristics[a]	Ice nucleation activity [$\log_{10}$ (ice nuclei per cell)] ± SD
JL4389	*lon*$^+$ *rpoS*$^+$	-1.7 ± 0.6
JL4594	*lon*::*aacC1* *rpoS*$^+$	-0.39 ± 0.1
JL4600	*lon*$^+$ *rpoS*::*lacZ*	-0.67 ± 0.1
JL4621	*lon*::*aacC1* *rpoS*::*lacZ*	0.17 ± 0.3
JL4601	*lon*$^+$ *rpoS*$^{++}$	-3.1 ± 0.4

[a] *rpoS*$^{++}$ designates the presence of both a genomic copy of *rpoS* and pJEL5649, a plasmid containing the cloned *rpoS* from Pf-5.

Lon accumulation increased after heat shock. Western analysis identified the Lon protein in Pf-5 (Fig. 2, lanes 2 to 5) but identified no detectable Lon in JL4292 (lane 10). When exponentially growing cultures of Pf-5 at an optical density at 600 nm (OD_{600}) of 0.2 (lane 2) were shifted from 27 to 42°C for 25 min to simulate heat shock (lane 3), Lon accumulation increased. In stationary-phase cultures at an OD_{600} of 2.0 (lane 4) or grown overnight to an OD_{600} of 2.0 to 4.0 (lane 5), Lon accumulation was not considerably greater than in exponentially growing cultures of Pf-5. An *rpoS*::Tn*5* mutant of Pf-5 still showed heat shock induction of Lon (lanes 6 and 7) and had higher levels of Lon than did Pf-5 in both the exponential (lane
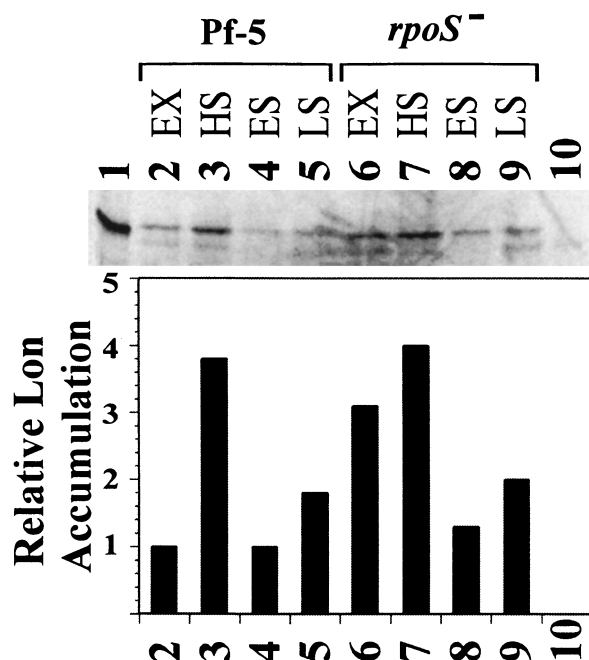

FIG. 2. Detection of Lon from Pf-5 grown in M9 minimal medium (45) with antiserum to Lon of *E. coli*. Sample numbers correspond to extracts from cells growing exponentially (EX) at 27°C at an OD_{600} of 0.2 (lanes 1, 2, and 6), cells heat shocked (HS) at 42°C for 25 min (lanes 3, 7, and 10), early-stationary-phase cultures (ES) at an OD_{600} of 2 (lanes 4 and 8), and cultures grown overnight to a final OD_{600} of 2 to 4 (late stationary phase [LS]) (lanes 5 and 9). Lanes: 1, *E. coli* SG20781; 2 to 5, Pf-5; 6 to 9, JL3985 (*rpoS*::Tn*5*); 10, JL4292 (*lon*::Tn*5*). Experiments were repeated with similar results. Scanned images were prepared for publication using Adobe Photo Shop version 4.0 (Adobe Systems Incorporated, San Jose, Calif.).
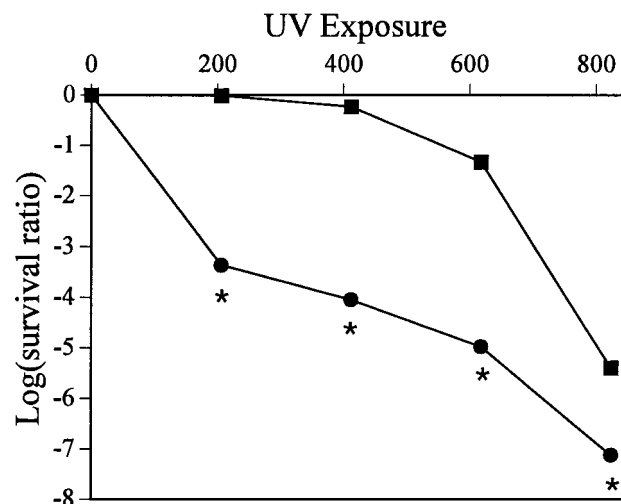

FIG. 3. Sensitivity of cells to UV irradiation. Stationary-phase cells of *P. fluorescens* Pf-5 (■) and JL4292 (●) were exposed to UV radiation ($\lambda = 254$ nm), expressed as ergs per square millimeter, for various durations. Colonies arising from surviving cells were counted following 48 h of incubation in the dark. Data were transformed as log survival ratio. Statistical analysis of variance was completed with SAS statistical software (Statistical Analysis Systems Institute, Cary, N.C.). Asterisks indicate data points where Pf-5 and JL4292 differ significantly ($P = 0.05$) as determined by Fisher's least-squares difference. The experiment was repeated with similar results.

6) and stationary (lanes 8 and 9) phases. Lon was detected and induced by heat shock in cells lacking GacS (data not shown). The amounts of σ^S in Pf-5 and the *lon*::Tn*5* mutant JL4292 were indistinguishable on these and other blots (data not shown). Results were similar when strains were grown in LB (data not shown).

***lon*::Tn*5* derivative was more sensitive than Pf-5 to UV irradiation.** We tested Pf-5 and its *lon*::Tn*5* derivative for their abilities to survive exposure to UV, because *lon* mutants of *E. coli* are more sensitive than *lon*$^+$ strains to UV irradiation (17, 18). The survival ratio of JL4292 was 1,000 times lower than that of Pf-5 after UV exposure at 206 erg/mm^2 and maintained that difference up to an exposure of 618 erg/mm^2 (Fig. 3). Cells of JL4292 were also noticeably elongated both before and after UV exposure, consistent with the lethal filamentation phenotype associated with *lon* in *E. coli*.

DISCUSSION

We cloned, sequenced, and partially characterized the *lon* homolog in *P. fluorescens* and demonstrated its role in the regulation of the antibiotic Plt. Like the stationary-phase sigma factor σ^S (46), Lon is a global regulator that negatively influences Plt production, so we evaluated the interactions of these regulators in Pf-5. Accumulation of Lon in cells of Pf-5 was not positively influenced by σ^S or GacS, a member of a two-component regulatory system that controls σ^S levels in this bacterium (53). Therefore, Lon does not appear to be an intermediate in the regulatory circuit involving GacA, GacS, and σ^S. Furthermore, levels of σ^S were similar in the *lon*::Tn*5* mutant and Pf-5, indicating that Lon and σ^S influence *plt* biosynthetic gene transcription and Plt production through separate regulatory circuits. It is possible that these circuits could converge through a *plt* pathway-specific regulator, which could integrate signals from diverse sensory transduction pathways.

Competition between sigma factors for limited core RNA polymerase is implicated in regulation of Lon accumulation (40) and Plt production (46, 47). In Pf-5, *lon* is preceded by

σ^{32}-like promoter sequences, suggesting that transcription of *lon* is initiated by the σ^{32} homolog σ^{H}. Furthermore, Lon accumulation increased after heat shock of Pf-5, as is typical of heat shock proteins transcribed from σ^{H} promoters. In an *rpoS*::Tn*5* derivative of Pf-5, Lon accumulation exceeded wild-type levels, indicating that the stationary-phase sigma factor σ^{S} negatively influences Lon. One possible explanation for this result is that expression of *lon* increases with the concentration of the σ^{H} RNA polymerase holoenzyme, which is likely to be enhanced in the absence of competing sigma factors such as σ^{S}. A precedent for this explanation exists in *E. coli*, in which the induction of heat shock proteins is observed in a strain with decreased levels of the housekeeping sigma factor σ^{70} (40). In light of recent evidence that Lon degrades σ^{H} in *Bacillus subtilis* under conditions in which the sigma factor has low activity (30), we considered the possibility that Lon repressed Plt production by degrading a sigma factor required for *plt* biosynthetic gene transcription. Close examination of the *plt* biosynthetic operon revealed no σ^{32}-like promoter sequences; therefore, it is unlikely that σ^{H} initiates *plt* transcription or that Lon represses *plt* transcription by degrading σ^{H} and reducing the amount of σ^{H} RNA polymerase holoenzyme. Previously, two other sigma factors were implicated in the regulation of Plt. Multiple copies of *rpoD*, encoding the housekeeping sigma factor σ^{D}, enhanced production of Plt in *P. fluorescens* strain CHA0 (47), a phenotype reminiscent of *rpoS* mutations in Pf-5 (46, 53). The identification of Lon as a regulator of antibiotic biosynthesis fits into an evolving model that proposes the involvement of multiple sigma factors in the regulation of Plt production (Fig. 4). Direct examination of the role of sigma factor competition in Plt regulation, not included in this study, is warranted by these and previous data.

An alternative mechanism for regulation of Plt by Lon is through degradation of a positive regulator of Plt production, as has been described for many phenotypes regulated by Lon (17, 18). For example, Lon degrades the transcriptional regulator RcsA, which, in association with the activator RcsB, positively regulates colonic acid capsular polysaccharide (*cps*) gene expression in *E. coli* (6, 17, 18). In the absence of Lon, RcsA has an enhanced half-life, resulting in overexpression of *cps* genes (17, 18). RcsA and other previously described targets of Lon degradation are not known to participate in regulatory circuits controlling Plt production in *P. fluorescens*. Nevertheless, Lon could repress Plt production by degrading an activator of *plt* biosynthetic gene transcription. We propose that one possible candidate for Lon degradation is PltR, a transcriptional activator that is linked to the *plt* biosynthetic operon (38). Comparisons between Lon substrates have failed to identify any likely consensus sequence, but motifs that are recognized by Lon may be defined by structure (18). Gottesman (17) has proposed that susceptibility of proteins to degradation is controlled by sequestration of target motifs within an active protein or protein complex. If a substrate of Lon that functions in activation of Plt production was identified and target motifs were characterized, the Plt activator could possibly be altered to be less susceptible to degradation by Lon. This could provide an opportunity to enhance Plt production by Pf-5 when *lon* is induced by certain stresses, consequently improving the activity of Pf-5 as a biological control agent.

In addition to the novel phenotype of antibiotic regulation, two phenotypes of *lon* mutants in *E. coli*, filamentation and enhanced UV sensitivity, were found in *lon* mutants of Pf-5. In *E. coli*, Lon specifically degrades SulA, a repressor of cell division that is induced during the cell's SOS response to severe DNA damage (35). When exposed to UV irradiation, *lon* mutants of *E. coli* accumulate SulA and consequently fail
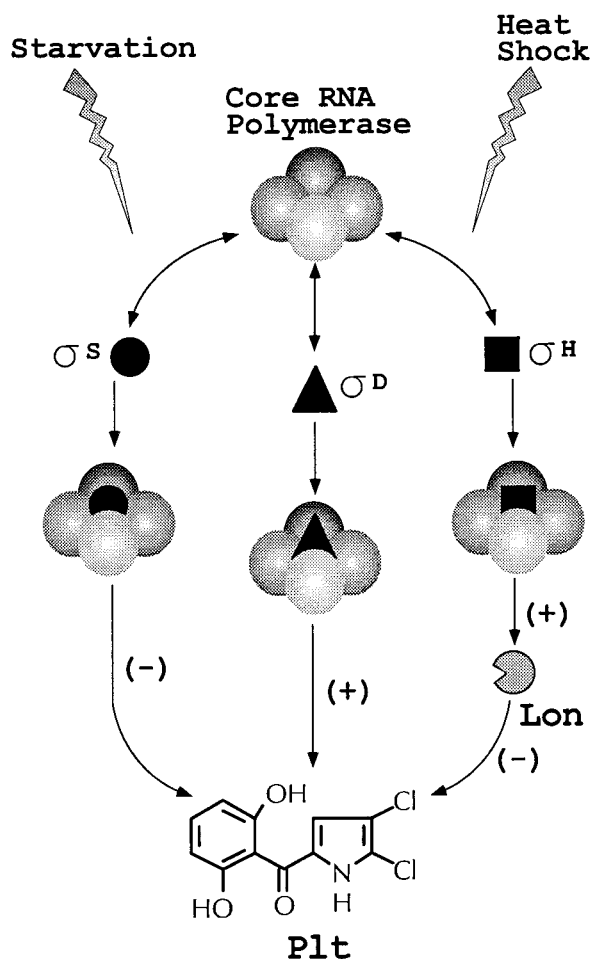

FIG. 4. Proposed model depicting regulation of Plt by several identified global regulators. On one level, competition between σ^{S}, σ^{D}, and σ^{H} for limited RNA polymerase core enzyme determines the suite of genes expressed and ultimately the level of Plt produced. In the absence of any one sigma factor, gene expression controlled by remaining sigma factors may increase due to decreased competition. In the presence of GacA and GacS, starvation rapidly induces σ^{S} expression (53), which negatively influences Plt production. In response to heat shock, σ^{H} mediates the induction of Lon, which negatively influences Plt production.

to divide, become filamentous, and die. Conservation of Lon function in regulating UV tolerance was observed among the two bacterial species, although direct involvement of SulA in *P. fluorescens* was not investigated.

The influence of *hupB*, located immediately downstream of *lon*, on antibiotic production by Pf-5 was also investigated in this study. Deleting the entire *hupB* gene reduced Plt production, increased Phl production, and resulted in a mucoid morphology. In *P. aeruginosa*, other histone-like proteins, including AlgP (22, 26) and integration host factor (36, 54), influence alginate production; similarly, the *hupB* product HU is implicated in the mucoidy phenotype of *E. coli* (41). We are uncertain why the Δ*hupB*::*aphI* derivative produced more Phl than Pf-5 or if Phl overproduction and mucoidy are related, but it is possible that increased culture viscosity influenced antibiotic production. Indeed, in *P. fluorescens* F113, researchers noted that increasing the viscosity of broth cultures by adding 1.5% agar increases Phl production by 10-fold (48). Alternatively, through its influence on DNA conformation, HU may influence transcription of genes required for antibiotic production.

Lon is the fourth global regulator in Pf-5, along with GacA,

GacS, and σ^S, that influences both stress response and antibiotic production by Pf-5. Both *lon::aacC1* and *rpoS::lacZ* mutants exhibit enhanced *pltB* transcription, implying that transcription of *plt* biosynthetic genes may be repressed under the stress conditions in which σ^H and σ^S are the dominant sigma factors directing transcription. Understanding the regulation of Plt production, both positive and negative, will allow manipulation of the strain to improve its consistency and performance as a biological control organism in the soil-root interface, where many stresses may be encountered.

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