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Involvement of the Lon Protease in the SOS Response Triggered by Ciprofloxacin in *Pseudomonas aeruginosa* PAO1

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Pseudomonas aeruginosa* PAO1 *lon* mutants have phenotypes of deficiencies in cell division, swarming, twitching, and biofilm formation as well as a phenotype of ciprofloxacin supersusceptibility. In this study, we demonstrated that a *lon* mutant was also supersensitive to the DNA-damaging agent UV light. To understand the influence of *lon* in causing these phenotypes, global gene expression was characterized by performing microarrays on the *lon* mutant and the PAO1 wild type grown in the presence of subinhibitory concentrations of ciprofloxacin. This revealed major differences in the expression of genes involved in the SOS response and DNA repair. Real-time quantitative PCR confirmed that these genes were highly upregulated upon ciprofloxacin exposure in the wild type but were significantly less induced in the *lon* mutant, indicating that Lon modulates the SOS response and consequentially ciprofloxacin susceptibility. As the known Lon target SulA is a member of the SOS response regulon, the influence of mutating or overexpressing this gene, and the negative regulator of the SOS response, LexA, was examined. Overexpression of *lexA* had no effect on the Lon-related phenotypes, but *sulA* overexpression recapitulated certain *lon* mutant phenotypes, including altered motility and cell division, indicating that Lon regulates these phenotypes through SulA. However, *sulA* overexpression did not affect ciprofloxacin susceptibility or biofilm formation, indicating that these properties were independently determined. Lon protease was also demonstrated to strongly influence RecA protein accumulation in the presence of ciprofloxacin. A model of DNA repair involving the Lon protease is proposed.

Pseudomonas aeruginosa is a versatile Gram-negative bacterium that is found in many natural environments (such as soils and marshes) but also causes infections of animals and plants (33). *P. aeruginosa* is a major opportunistic human pathogen, being the third most common cause of nosocomial infections. It can cause pneumonia, urinary tract infections, and bacteremia, as well as morbidity and mortality in cystic fibrosis patients due to chronic infections that eventually lead to lung damage and respiratory failure. *Pseudomonas* infections are difficult to eradicate due to high intrinsic resistance of the bacterium, together with its ability to develop resistance to common antibiotics through adaptation and mutation. Multidrug (especially including imipenem)-resistant strains of *P. aeruginosa* have joined the ranks of "superbugs." Ciprofloxacin, a fluoroquinolone, is one of the antibiotics currently used for treatment of *P. aeruginosa* infections (12). Fluoroquinolones target the bacterial enzymes DNA gyrase and topoisomerase IV (10), which are essential for correct DNA replication and transcription. However, the overuse of fluoroquinolones has led to the rise and spread of quinolone resistance in *P. aeruginosa* strains (39).

Screening of the PAO1 mini-Tn*5*–*luxCDABE* (22) and PA14 Harvard (23) mutant libraries, for altered susceptibility to ciprofloxacin revealed that dozens of genes are involved in intrinsic and mutational resistance to this antibiotic (2, 4). One gene of interest was *lon*, which encodes the Lon protease. Lon is an ATP-dependent cytoplasmic serine protease which associates into hexameric rings in Gram-negative bacteria. It is a homo-oligomer composed of an N-terminal domain, an ATP binding domain, a substrate sensor and discriminatory domain, and a proteolytically active C-terminal domain (5). Lon belongs to the group of the self-compartmentalized or chambered proteases. The Lon protease from *P. aeruginosa* has 84% similarity to the Lon protease of *Escherichia coli* (www.pseudomonas.com). Studies on the *E. coli* Lon protease have shown that Lon degrades the antitermination protein (N protein) of phage λ and is involved in the lysogenic switch of lambda (20, 26). Furthermore, Lon is involved in unfolding misfolded proteins during heat shock as well as in their degradation. Other known Lon targets in *E. coli* include SulA, which regulates cell division (28), and RcsA, a transcriptional activator for capsule synthesis (35). *E. coli lon* mutants demonstrate filamentation, sensitivity to UV light and DNA damage, as well as fluoroquinolone susceptibility (40).

The role of Lon in *P. aeruginosa* has not yet been studied in depth, but it appears to have the appropriate characteristics to be a central player in the complex adaptations of this organism. To date, it is known that *lon* mutants of *P. aeruginosa* show ciprofloxacin supersusceptibility, filamentation (2), and deficiencies in swarming motility, twitching motility, and biofilm formation (25). Furthermore, the ATP-dependent Lon protease is a negative regulator of quorum sensing, and a *lon* mutant exhibits increased hemolytic activity (34). The *lon* gene is induced by treatment with subinhibitory aminoglycosides (25) but not in wild-type strains in the presence of subinhibitory concentrations of ciprofloxacin.

In this study, we investigated the mechanisms by which Lon participates in the regulation of ciprofloxacin resistance in *P. aeruginosa*. Our results indicate that the SOS response triggered by DNA-damaging agents is suppressed in a *lon* mutant compared to in the wild type. This explained the increased sensitivity to fluo-

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TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Description/characteristic(s)[a]	Reference
Strains		
P. aeruginosa		
H103 (wild type)	Wild-type *P. aeruginosa* PAO1 strain H103	Lab collection
lon (PA1803) mutant	PAO1 mini-Tn*5*–*luxCDABE*::*lon*; 74_D9, Tet^r	22
recA (PA3617) mutant	PAO1 mini-Tn*5*–*luxCDABE*::*recA*; 69_C12, Tet^r	22
lexA (PA3007) mutant	PA3007::IS*phoA*, Tet^r, derived from UW-WT	16
sulA (PA3008) mutant	PA3008::IS*lacZ*, Tet^r, derived from UW-WT	16
lonc	PAO1 *lon*::mini-Tn*5*–*luxCDABE* (pBBR1MCS4:*lon*+), Cb^r	This study
sulA-overexpressing strain	PAO1(pBBR1MCS4::*sulA*$^+$), Cb^r	This study
lexA-overexpressing strain	PAO1(pBBR1MCS4::*lexA*$^+$), Cb^r	This study
E. coli		
TOP10	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 recA1 ara*Δ*139* Δ(*ara-leu*)*7697 galU galK rpsL* (Str^r) *endA1 nupG*	Invitrogen
DH5α	F$^-$ ϕ80*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17*(r_K^- m_K^+) *supE44* λ$^-$ *thi-1 gyrA96 relA*	Invitrogen
Plasmids		
pCR-Blunt II-TOPO	PCR cloning vector; Kan^r	Invitrogen
pBBR1MCS4	Broad-host-range cloning vector; Amp^r	21
pBBR1MCS4:*lon*+	Cloning vector harboring *lon* amplicon; Amp^r	This study
pBBR1MCS4:*sulA*+	Cloning vector harboring *sulA* amplicon; Amp^r	This study
pBBR1MCS4:*lexA*+	Cloning vector harboring *lexA* amplicon; Amp^r	This study

[a] Antibiotic resistance phenotypes: Amp^r, ampicillin resistance for *E. coli*; Cb^r, carbenicillin resistance for *P. aeruginosa*; Kan^r, kanamycin resistance; Tet^r, tetracycline resistance.

roquinolones and UV light in the absence of the Lon protease, and here we propose a model for that regulation.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains used in this study are shown in Table 1. Growth was routinely performed in Luria-Bertani broth (LB) unless indicated otherwise. For assessment of growth, overnight cultures of strains were diluted 1:100 into fresh LB and monitored by using a Tecan Spectrafluor Plus for 24 h by measuring the absorbance at 620 nm every 20 min.

MIC. The standard broth microdilution method was used for measurement of the MIC of ciprofloxacin in LB (38).

Assessing killing by ciprofloxacin. Cells of *P. aeruginosa* were grown to mid-log phase (optical density at 600 nm [OD_{600}] of 0.5) in LB medium. The cultures were pelleted by centrifugation (3,000 × *g* for 10 min) and resuspended in 1× BM2 minimal salts [62 mM potassium phosphate buffer, pH 7.0, 7 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 10 μM $FeSO_4$], and 1× MIC of ciprofloxacin was added to the samples. Samples were shaken at 37°C, and aliquots were taken at specified times up to 90 min after antibiotic addition, plated for survivors on LB agar, and grown overnight at 37°C. All experiments were repeated at least three times.

DNA microarray experiment. Each microarray experiment was performed as previously described (27), comparing five independent cultures of the *P. aeruginosa* PAO1 wild type and the *lon* mutant. The cultures were grown to mid-logarithmic phase (OD_{600} of 0.5) with subinhibitory (one-half the MIC) concentrations of ciprofloxacin (0.05 μg/ml for the wild type and 0.0125 μg/ml for the mutant). Briefly, RNA was isolated, reverse transcribed into cDNA, and then labeled with Cy3 or Cy5 dye. Labeled sample pairs were applied to a spotted microarray provided by the J. Craig Venter Institute, hybridized overnight, and scanned on a ScanArray Express scanner and analyzed using Imagene software and ArrayPipe version 1.7. Only statistically significant changes in gene expression were considered here. Changes in gene expression for several genes of interest were confirmed using real-time quantitative PCR (RT-qPCR).

Real-time quantitative PCR. The ABI Prism 7000 sequence detection system together with SYBR green I dye (Applied Biosystems) was used to perform RT-qPCR to validate microarray data. For primer design, the ABI Primer Express program (v2.0) and the gene sequence derived from the *Pseudomonas* database (www.pseudomonas.com) were used. cDNA was prepared as described above.

UV irradiation sensitivity assay. To test UV sensitivity, 200 μl of an overnight culture was diluted 1:10 in LB and grown for 4 h at 37°C with shaking. After 4 h, the culture was diluted again 1:10 and transferred to a petri dish. UV irradiation was performed with a Chemigenius2 Bio imaging system, which contains 10 UV lamps, from Syngene. The total power was 16 W, the radiant energy was 12 W, and the sample was placed at a distance of 0.5 inches from the UV lamp. The sample was exposed to the UV light for 10 s, after which it was serially diluted and plated on LB agar. The number of CFU was counted the next day. The assay was repeated in triplicate.

Overexpression studies. The low-copy-number vector pBBR1MCS4 contains a *lac* promoter just upstream of the multiple-cloning site (MCS) (21). For overexpression studies, the respective genes *lexA* (PA3007) and the *sulA* homolog (PA3008) were PCR amplified and cloned into TopoBlunt. This was followed by restriction digestion with the enzymes HindIII and XbaI. These fragments were then ligated into pBBR1MCS4 and transformed into *E. coli* DH5α competent cells. After plasmid isolation and transformation into wild-type *P. aeruginosa* PAO1, the constructs were checked by PCR, sequencing, and assessment of appropriate size after plasmid isolation. Experiments performed include RNA isolation, growth curve, MIC, swarming, swimming, twitching, and biofilm formation experiments.

Motility assays. Swarming motility was measured as described previously (29) by inoculating 1 μl of a mid-logarithmic-phase bacterial culture onto a swarm plate containing modified BM2 minimal medium, containing 0.5% Casamino Acids instead of 7 mM ammonium sulfate and 0.5% agar. Swarming behavior was compared to that of the wild type after 20 h of incubation at 37°C. Swimming motility was evaluated on BM2 plates containing 0.3% agar; 1 μl of a culture was inoculated on a swimming plate, and the resultant diameters of the swim zones were measured after 20 h of incubation at 37°C. The ability of *Pseudomonas* strains to twitch on a solid surface or interface was evaluated by stabbing 1 μl of an overnight culture through a 1% agar plate to the interface between the

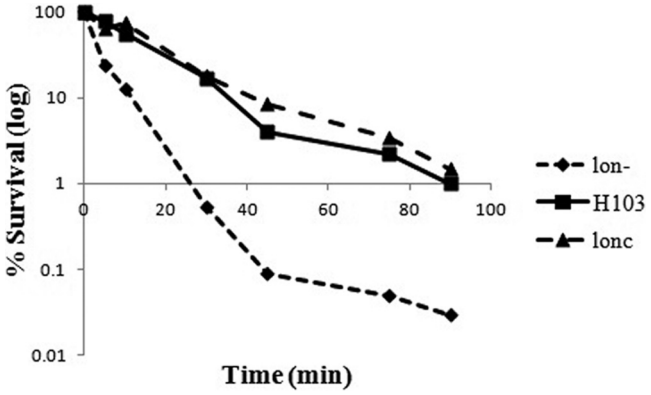

FIG 1 Ciprofloxacin-mediated killing of *P. aeruginosa* at 1× MIC. Cultures of *P. aeruginosa* wild-type strain H103, the *lon* mutant, and the complemented mutant (*lonc*) were grown to mid-log phase, washed, and then exposed to 0.1 μg/ml ciprofloxacin (1× MIC). After plating and overnight incubation, residual CFU were determined over an incubation period of 90 min. Results from 1 out of three representative experiments are shown.

plastic and the bottom of the agar. The twitching zone was measured after 24 h of incubation at 37°C.

Biofilm assay. An abiotic solid surface assay was used to measure mature biofilm formation and has been previously described (11). Overnight cultures were diluted 1:100 in LB and inoculated into 96-well polystyrene microtiter plates and incubated at 37°C for 20 h without shaking. Crystal violet was used to stain the biofilms, and the absorbance was measured at 595 nm with a microtiter plate reader (Bio-Tek Instruments).

Mutagenesis experiments. The formation of resistant colonies induced by ciprofloxacin was studied. Briefly, bacterial overnight cultures were diluted to an OD_{600} of 0.1 in 0.7% NaCl before they were spread onto an LB plate. A hole was punched in the middle of the agar plate, and it was filled with a total of 5 μg of ciprofloxacin. Plates were incubated at 37°C for 48 to 72 h, and the resistant colonies formed in the clearance zone were counted. The assay was repeated in 3 independent experiments.

Protein gel and Western blot. Whole bacterial cells were denatured by boiling at 100°C for 5 min in 2× protein solubilization buffer (0.5 M Tris [pH 6.8], glycerol, 10% SDS, 0.5 M EDTA, distilled water [dH_2O]). Denatured samples were then separated on a 12% SDS-PAGE gel. Gels were stained overnight in Coomassie blue. Western blotting was carried out by transferring the protein bands from an unstained SDS-polyacrylamide gel to a polyvinylidene fluoride (PVDF) membrane (Amersham) by using a tank blot apparatus, blocked for 1 h in TBS (20 mM Tris, pH 7.4, 150 mM NaCl) containing 5% skim milk powder and 1% bovine serum albumin (BSA), and incubated with a rabbit polyclonal antibody against *E. coli* RecA protein (1:1,000; Abcam) (this antibody recognizes RecA in multiple species due to the high homology of the forms of this protein between species) overnight at 4°C in TBS plus 5% skim milk powder. Membranes were washed 8 times for 10 min in TBST (TBS plus 0.1% Tween 20) and then incubated with anti-rabbit IgG horseradish peroxidase (HRP)-linked (1:3,000; Cell Signaling Technology) antibody (secondary antibody) for 30 min in TBS plus 5% skim milk powder. Washing the membranes 5 times for 10 min in TBST allowed for the removal of unbound secondary antibody. The ECL kit (Amersham) was used to develop protein bands.

Microarray accession number. The ArrayExpress accession number corresponding to this work is E-MTAB-1000.

RESULTS

Supersusceptibility phenotype. In agreement with previous data (2), MIC measurements showed that the *lon* mini-Tn*5*–*luxCDABE* mutant displayed a 4-fold increased susceptibility to ciprofloxacin compared to the wild type and the complemented strain. This supersusceptibility phenotype was also evident when susceptibility was analyzed by means of killing curves. After 10 min of killing in the presence of 1× MIC (0.1 μg/ml) ciprofloxacin, the *lon* mutant, the complemented strain, and the wild type showed survival rates of 10%, 70%, and 60%, respectively (Fig. 1). The same trend was observed when killing was assessed at 2× MIC. The increased susceptibility of the mutant compared to that of the wild type was even more accentuated at later time points in that after 45 min, only 0.1% of the *lon* mutant cells survived exposure to ciprofloxacin, whereas the wild type and the complemented strain showed survival rates of around 5 to 10%.

Microarray analysis of the *P. aeruginosa lon* mutant under subinhibitory concentrations of ciprofloxacin. To understand the molecular basis for the ciprofloxacin supersusceptibility phenotype displayed by *lon* mutants, gene expression was investigated during growth in subinhibitory concentrations of ciprofloxacin in order to trigger any adaptive mechanisms. Microarray analysis revealed that in the presence of ciprofloxacin, there was differential expression of 230 genes between the mutant and the wild type (~4% of all *P. aeruginosa* genes) using a cutoff of >2-fold change and a *P* value of <0.05 (see Table 2 for selected genes and Table S1 in the supplemental material for a complete list of dysregulated genes). Genes involved in the SOS response, including *recA*, *lexA*, *sulA*, *recN*, *prtR*, and *prtN*, and the heat shock genes (*dnaK*, *hslV*, *hslU*, and *groES*) are known to be upregulated by ciprofloxacin in the wild type (3). Here we demonstrated that, except *groES*, all of these genes were less expressed in the *lon* mutant under the conditions utilized here (Tables 2 and 3). Furthermore, 13 out of the 15 LexA-regulated genes (*phl*, *dnaE2*, *imuB*, *sulA2*, PA0922, PA1044, *dinG*, PA2288, *lexA*, *sulA*, *yebG*, PA3414, *recX*, *recA*, and

TABLE 2 Selected *P. aeruginosa* genes differentially expressed in the *lon* mutant in the mid-log-phase microarray under subinhibitory (one-half MIC) concentrations of ciprofloxacin

PA no.	Gene name	Gene function/product	Mid-log phase	
			Fold change[a]	*P* value
PA0069		Conserved hypothetical protein	−5.5	0.00
PA0610	*prtN*	Transcriptional regulator PrtN	−12.7	0.00
PA0611	*prtR*	Transcriptional regulator PrtR	−3.0	0.00
PA0625		Hypothetical protein in phage pyocin operon	−5.6	0.00
PA0649	*trpG*	Anthranilate synthase component II	−3.2	0.00
PA0650	*trpD*	Anthranilate phosphoribosyltransferase	−3.5	0.01
PA0651	*trpC*	Indole-3-glycerol-phosphate synthase	−2.4	0.00
PA0669		Probable DNA polymerase alpha chain	−3.1	0.00
PA0670		Hypothetical protein	−5.3	0.00
PA0671	*sulA2*	Hypothetical protein	−5.6	0.00
PA0865	*hpd*	4-Hydroxyphenylpyruvate dioxygenase	2.8	0.00
PA0872	*phhA*	Phenylalanine-4-hydroxylase	2.8	0.00
PA0922		Hypothetical protein	−2.4	0.00
PA2008	*fahA*	Fumarylacetoacetase	3.0	0.00
PA2009	*hmgA*	Homogentisate 1,2-dioxygenase	8.1	0.00
PA2288		Hypothetical protein	−2.1	0.00
PA3007	*lexA*	Repressor protein LexA	−2.2	0.01
PA3413		Hypothetical protein	−2.4	0.00
PA3414		Hypothetical protein	−2.7	0.00
PA3616	*recX*	Conserved hypothetical protein	−2.2	0.00
PA4761	*dnaK*	DnaK protein	−2.1	0.01
PA5053	*hslV*	Heat shock protein	−5.0	0.00
PA5054	*hslU*	Heat shock protein	−2.3	0.00

[a] A negative fold change represents reduced upregulation, whereas a positive change represents increased upregulation.

TABLE 3 Expression of selected genes involved in SOS response and DNA damage repair under various conditions

PA no.	Gene name	Gene function/product	Fold change in gene expression[a]			
			WT sub-cipro vs WT	*lon* mutant vs WT	*lon* mutant sub-cipro vs *lon* mutant	*lon* mutant sub-cipro vs WT sub-cipro
PA0610	*prtN*	Transcriptional regulator PrtN	11.08 ± 2.85	2.40 ± 0.14	0.77 ± 0.29	0.15 ± 0.03
PA0611	*prtR*	Transcriptional regulator PrtR	4.06 ± 0.72	1.65 ± 0.36	0.84 ± 0.03	0.33 ± 0.00
PA0625		Hypothetical protein	28.40 ± 8.13	2.95 ± 0.16	1.30 ± 0.30	0.16 ± 0.05
PA3007	*lexA*	Repressor protein LexA	9.01 ± 0.81	0.99 ± 0.22	3.27 ± 0.76	0.35 ± 0.05
PA3008	*sulA*	Hypothetical protein	9.35 ± 1.77	1.38 ± 0.34	3.42 ± 0.46	0.50 ± 0.09
PA3617	*recA*	RecA protein	6.07 ± 1.68	1.29 ± 0.06	2.35 ± 0.65	0.50 ± 0.00
PA4763	*recN*	DNA repair protein RecN	14.85 ± 2.35	0.83 ± 0.25	5.44 ± 1.85	0.29 ± 0.06

[a] Each fold change is represented as the average of 3 biological repeats with the standard deviation (the first listed element relative to the second listed element). WT, wild type; sub-cipro, subinhibitory concentration of ciprofloxacin.

recN) identified by Cirz et al. (6) were less expressed in the *lon* mutant under the conditions used here (Table 2; see Table S1 in the supplemental material). This further emphasized that the SOS damage response is majorly impacted in a *lon* mutant.

The microarray also revealed that in the presence of subinhibitory concentrations of ciprofloxacin, Lon was a repressor of the phenylalanine degradation pathway and was likely affecting anthranilate synthesis in the presence of ciprofloxacin based on the dysregulation in the *lon* mutant of the following genes: *phhA*, *hpc*, *hpd*, *hmgA*, *fahA*, *trpG*, *trpD*, and *trpC*.

Role of Lon in the SOS response in the presence of ciprofloxacin. To further investigate if some of the dysregulated genes mentioned above were responsible for the supersusceptible phenotype of the *lon* mutant, we determined whether selected genes were dysregulated to a lesser extent in the *lon* mutant than in the wild type in the presence of subinhibitory concentrations of ciprofloxacin.

The expression levels of selected genes were determined by RT-qPCR, comparing the *lon* mutant strains with and without subinhibitory concentrations of ciprofloxacin as well as the wild-type strains with and without subinhibitory ciprofloxacin. This demonstrated that the induction of genes involved in the SOS response and DNA repair was indeed significantly impaired in the *lon* mutant, as this mutant showed only a minor response or no response in the presence of ciprofloxacin, compared to the situation in the wild type. For example, *recA*, a major SOS response gene, was upregulated 6.1-fold in the wild type in the presence of ciprofloxacin; however, the mutant showed just a 2.4-fold increase in the transcription of this gene. The differential induction of the expression of genes involved in the SOS response and DNA repair was also shown (Table 3) for *lexA*, *sulA*, and *recA* as well as *prtN*, *prtR*, and the entire RecA/PrtR/PrtN-regulated phage pyocin region, e.g., PA0625, which is involved in ciprofloxacin susceptibility (3).

This is consistent with the explanation that subinhibitory ciprofloxacin caused DNA damage in the wild type, eliciting an SOS response in an attempt to repair this damage, but that the *lon* mutant was substantially deficient in the induction of this response. We thus suggest that this limited inducibility of the SOS DNA repair responses in the *lon* mutant explains its observed phenotype of supersusceptibility to the DNA-damaging agent ciprofloxacin, consistent with previous observations that mutants in the *recA*, *recN*, and *recG* genes showed increased supersusceptibility to ciprofloxacin and/or UV light (2, 18). Intriguingly, Brazas et al. (3) previously reported that RecA controlled the expression of a large phage pyocin operon in response to ciprofloxacin and that this influenced susceptibility to ciprofloxacin. Here, we were able to demonstrate that genes in this operon, e.g., PA0625 (and the direct regulators *prtRN*), were substantially downregulated (Table 2), although this would actually counteract ciprofloxacin susceptibility in the *lon* mutant.

Effect of Lon on ciprofloxacin-mediated stimulation of mutagenesis. Ciprofloxacin is known to be a good inducer of mutagenesis, as it interferes with DNA replication (6). The importance of the Lon protease in this mutagenic activity was evaluated. In a plate assay, ciprofloxacin (total, 5 μg) was used to induce mutagenesis, and the ability of cells to grow in the clearing zone was evaluated for the wild type, the *lon* mutant, and the complemented strain. The results demonstrated that Lon was necessary for the induction of mutagenesis, since 2 ± 1 resistant colonies were observed in the case of the *lon* mutant, whereas 77 ± 8 and 61 ± 11 colonies were observed for the wild type and complemented strain, respectively (Fig. 2). These re-

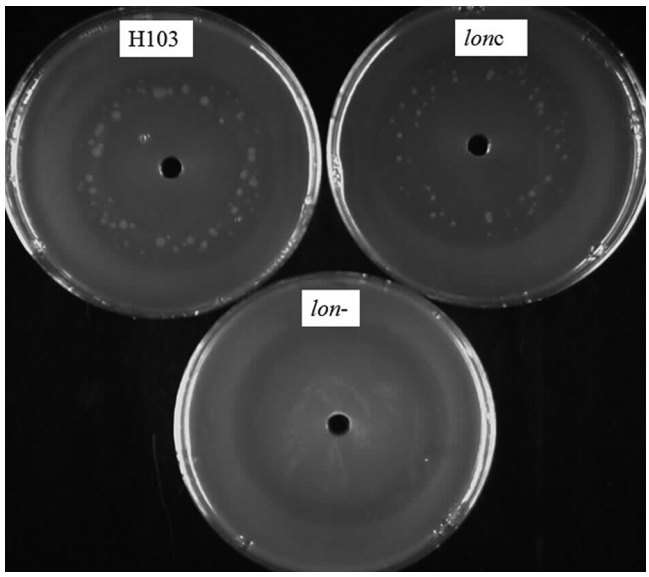

FIG 2 Effect of Lon on ciprofloxacin-mediated stimulation of mutagenesis. Cultures of *P. aeruginosa* wild-type strain H103, the *lon* mutant, and the complemented mutant (*lonc*) were diluted to an OD_{600} of 0.1 and spread onto an LB plate. A hole was punched in the middle of the agar plate, and it was filled with a total of 5 μg of ciprofloxacin. Plates were incubated at 37°C for 48 to 72 h, and the resistant colonies formed in the clearing zone were counted. Results from one of three representative experiments are shown.

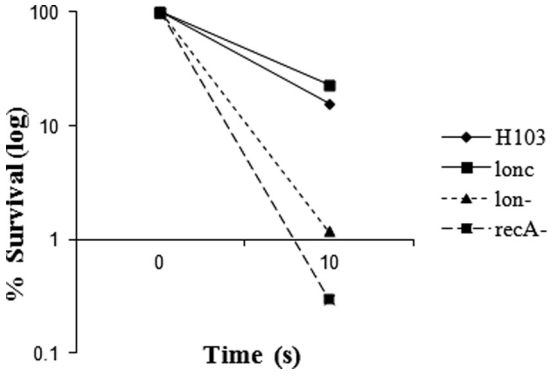

FIG 3 UV-mediated killing of *P. aeruginosa*. Cultures of *P. aeruginosa* wild-type strain H103, the *lon* mutant, the complemented mutant (*lonc*), and the *recA* mutant were grown to mid-log phase, diluted, and then exposed to UV light for 10 s and residual CFU determined. Results from one of three representative experiments are shown.

sults were consistent with an effect of Lon on DNA repair through the SOS system.

The *lon* mutant demonstrated increased sensitivity to UV irradiation. Like ciprofloxacin, UV light is a DNA-damaging agent, and thus, we predicted that the *lon* mutant would be more sensitive to UV killing than the wild type, as previously shown for *lon* mutants of *E. coli* and *P. fluorescens* Pf-5 (13, 14, 37). For the *P. aeruginosa lon* mutant, a 20-fold increase in susceptibility to UV irradiation was demonstrated after 10 s of UV light exposure. This susceptibility phenotype could be reversed by complementation. A mini-Tn*5*–*luxCDABE recA* mutant (22) was included as a control of the experimental conditions, confirming the known susceptibility of *recA* mutants to UV damage (Fig. 3) (18).

Dysregulation of *lon* during lethal UV light and ciprofloxacin exposure. Treatment of the PAO1 wild-type strain of *P. aeruginosa* with either a lethal dose of UV light (30 s exposure) or 4× MIC (0.4 μg/ml) of ciprofloxacin resulted in a moderate downregulation of *lon* expression (0.25 ± 0.16-fold and 0.42 ± 0.20-fold, respectively); conversely, a gene involved in the SOS response (*lexA*) was highly upregulated ([8 ± 2.57]-fold) and served as an experimental control.

Phenotypic effects of *sulA* and *lexA* overexpression. LexA is the primary repressor of the SOS DNA damage response and a target of RecA coprotease (24). SulA acts downstream of LexA and RecA in the SOS response to repress cell division while damage is being repaired (15). For *E. coli*, it has been shown to be a direct target for the protease action of Lon (28). If Lon were acting directly on either the LexA or SulA protein in *P. aeruginosa*, then removal of Lon by mutation would have the effect of increasing its function, which we attempted to mimic by overexpressing the cloned genes. Overexpression of *sulA* (PA3008) and *lexA* (PA3007) was achieved by constitutive expression of the cloned genes behind the *lacZ* promoter (constitutive in *Pseudomonas*) of the low-copy-number vector pBBR1MCS4. The expression level was checked by RT-qPCR and revealed a 31-fold upregulation for the *sulA*-overexpressing clone compared to the vector alone and 101-fold upregulation in the case of the *lexA*-overexpressing clone. Overexpression of *sulA* or *lexA* did not result in any major growth defect as measured using the Tecan Spectrafluor Plus. Cells overexpressing LexA showed a normal swarming behavior like the wild type and a *lexA* mutant. In contrast, overexpression of *sulA* led to a swarming deficiency similar to that exhibited by a *lon* mutant, whereas a *sulA* mutant did not have a swarming defect. The same observations were made for the other motilities affected by Lon, namely, swimming (mediated by flagella in an aqueous environment) and twitching (on solid surfaces and interfaces mediated by type IV pili). No major changes in ciprofloxacin MIC or biofilm formation were noted for the constructs overexpressing either *sulA* or *lexA* compared to the control, even though biofilm formation is another form of social behavior mediated by flagella and type IV pili. As expected, the strain overexpressing *sulA* formed, like a *lon* mutant, long filaments, since SulA is a cell division inhibitor (Fig. 4). This is consistent with the explanation that Lon degrades SulA also in *P. aeruginosa* and that *lon* mutants likely accumulate SulA. Accumulation of SulA in cells would lead to the formation of long filaments, and such cells might demonstrate motility defects. However, SulA accumulation clearly did not affect other Lon phenotypes.

To investigate if Lon amplified the SOS response through SulA, transcriptional regulation was investigated for the strain overexpressing *sulA*. The *sulA*-overexpressing strain showed a transcriptional profile for the genes *recA* and *lexA*, in the presence of subinhibitory concentrations of ciprofloxacin, that was similar to the transcriptional profile of the control. Under nondamaging conditions, *sulA* was, as expected, highly upregulated in the strain overexpressing *sulA*. However, in the presence of DNA damage, *sulA* was less expressed in the overexpressing strain, indicating the autoregulation of *sulA* itself (Table 4).

Effect of ciprofloxacin on protein expression in the *lon* mutant. Since no apparent difference in transcriptional regulation was observed for the *sulA*-overexpressing strain, we hypothesized that the Lon protease influenced protein accumulation in the presence of ciprofloxacin. Whole-cell lysates were used to determine if Lon impacted the major protein of the SOS response (RecA) under DNA-damaging conditions, such as subinhibitory concentrations of ciprofloxacin. SDS-polyacrylamide gel electrophoresis and Western blot analysis demonstrated that expression of RecA (37 kDa) was induced under subinhibitory concentrations of ciprofloxacin in the wild type and the complemented *lon* strain; however, it failed to be expressed in the *lon* mutant, thus highlighting that RecA production was dependent on the Lon protease (Fig. 5). Under noninducing conditions, no RecA band was visible in any of the strains tested, as RecA is accumulated only upon DNA damage.

DISCUSSION

This study demonstrates for the first time that the ATP-dependent Lon protease (PA1803) plays a major role in regulating the SOS response and DNA repair in *P. aeruginosa*. Studies on the ATP-dependent Lon protease in different bacteria have shown its involvement in such diverse processes as cell division (2, 30), flagellar biosynthesis (31), capsule synthesis (35), UV tolerance (37), motility (25), and antibiotic resistance (2). However, the phenotypes of the *lon* mutants vary between different bacterial species. In *P. aeruginosa* PAO1, *lon* mutants exhibit a motility defect, show extreme filamentation and greater hemolytic activity, and of particular interest, are supersusceptible to ciprofloxacin (2, 25, 34). Explaining this supersusceptibility was of great interest to us.

Previous studies showed that subinhibitory and inhibitory concentrations of ciprofloxacin lead to dramatic changes in global transcription. Most striking were the upregulation of a susceptibility determinant, the R2/F2 pyocins (PA0613 to PA0648), and the genes involved in DNA repair and SOS response (3).

Overall, our microarray and RT-qPCR results showed that the

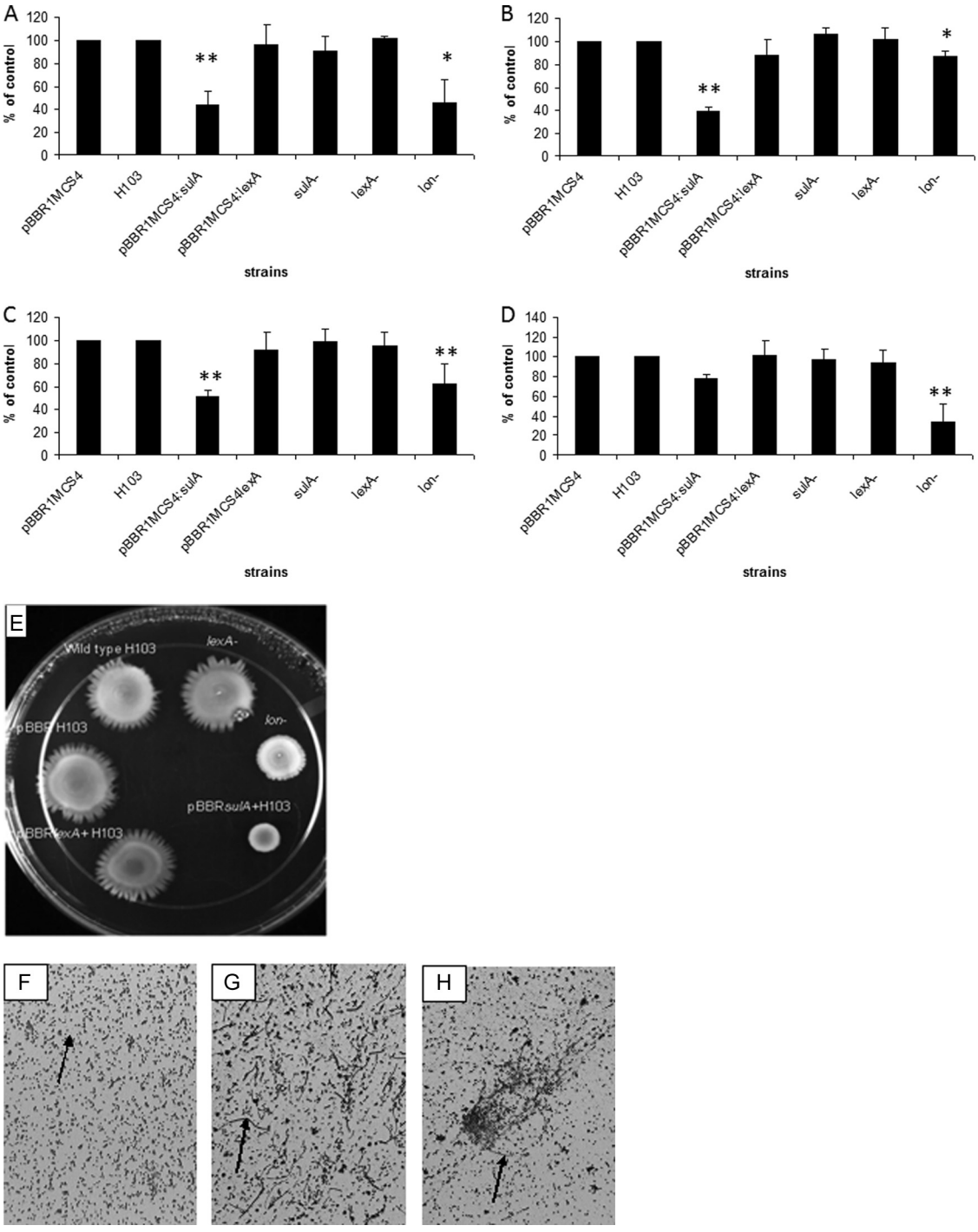

FIG 4 Effect of *sulA* and *lexA* overexpression on various types of motility. Cultures of *P. aeruginosa* overexpressing either *lexA* or the *sulA* homolog (PA3008) were grown to mid-log phase and then inoculated onto swarming, swimming, or twitching plates. Swarming motility (A and E), swimming (B), twitching (C), and biofilm formation (D) were assayed compared to the wild type. The filamentation phenotype was analyzed by light microscopy. The wild type (F), the *sulA*-overexpressing strain (G), and the *lon* mutant (H) are shown. The bars and error bars represent the averages and standard deviations of 3 independent experiments. A statistically significant difference was observed for the *sulA*-overexpressing strain at the indicated cases with a *P* value of ≤0.003 (Student's *t* test, indicated by **; for lon- the *P* value of <0.05 is indicated by *).

Lon protease of *P. aeruginosa* was important for full induction of the SOS response upon exposure to DNA-damaging agents, including ciprofloxacin. Brazas et al. (2) previously demonstrated that preexposure to ciprofloxacin led to adaptive resistance in the *P. aeruginosa* wild-type PAO1 but failed to induce adaptive resistance in the *lon* mutant. It was concluded that the extreme cell elongation phenotype of the mutant contributed to this phenotype. In the present study, we were able to demonstrate another

TABLE 4 Effects on SOS transcriptional regulation of the *sulA*-overexpressing strain compared to the vector control after subinhibitory ciprofloxacin induction

Gene	Fold change in gene expression[a]: Vector control plus ciprofloxacin[b] vs vector control (no antibiotic)	Fold change in gene expression[a]: *sulA*-overexpressing strain plus ciprofloxacin vs *sulA*-overexpressing strain
recA	4.0 ± 1.5	5.0 ± 1.9
lexA	5.9 ± 0.8	11.0 ± 4.1
sulA	4.3 ± 1.3	1.4 ± 0.9

[a] Fold changes represent the increase in the first listed element relative to the second listed element.
[b] Induction was with a subinhibitory concentration of ciprofloxacin.

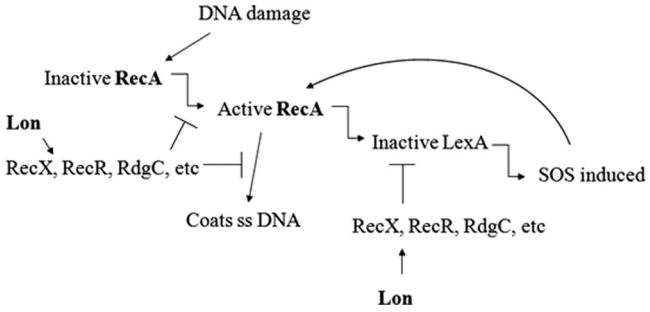

FIG 6 Proposed model for the involvement of the Lon protease in the DNA damage response. Under DNA-damaging conditions, the Lon protease is proposed to cleave and thus antagonize one or more of RecX, RecR, and RdgC, etc., which are known repressors of RecA in *E. coli* (8, 9, 32, 36). This inhibition leads to autoamplification of RecA, and the SOS response is induced. However, if the Lon protease is inactive, the repressors are proposed to inhibit RecA function and autoamplification.

explanation, namely, the weaker induction of the SOS response and DNA repair observed in the *lon* mutant. The cell elongation phenotype triggered through the SOS response is controlled by SulA (17), and overexpression of *sulA* did not recapitulate ciprofloxacin susceptibility, indicating that elongation likely did not explain altered ciprofloxacin susceptibility. The negative and the positive regulators of the SOS response, LexA and RecA, respectively, are common in all bacterial species and are highly upregulated in *P. aeruginosa* in response to environmental stress. While these genes and several other genes involved in the SOS response are upregulated in the wild type after ciprofloxacin treatment to overcome the DNA damage, the present study demonstrated that if *lon* was mutated, this SOS response was considerably weaker. Consistent with this deficiency in this DNA repair system, it was demonstrated that Lon protease was important in ciprofloxacin-mediated mutagenesis. Some SOS response genes were still upregulated by ~3-fold in the *lon* mutant, but this induction was substantially lower than in the wild type. We propose a model (Fig. 6) whereby Lon protease normally inhibits the action of RecA repressors (RecX, RecR, and RdgC, etc.) (8, 9, 32, 36), leading to the autoamplification of RecA. In the situation where Lon is inactive, the repressor stays intact and no RecA amplification would occur, leading to supersusceptibility to DNA-damaging agents, such as fluoroquinolones and UV light, as damage cannot be repaired. Consistent with this, mutants in genes involved in the SOS response and DNA repair (*recA*, *recN*, and *recG*) exhibit an increased susceptibility to ciprofloxacin and UV light (2, 18).

Under normal conditions, the LexA repressor binds to the SOS box located in the promoter region of genes involved in the SOS response and represses the expression of genes involved in DNA repair. The SOS response includes 43 genes in *E. coli* (7), 33 genes in *Bacillus subtilis* (1), and at least 15 genes in *P. aeruginosa* (6). Upon DNA damage, the resulting single-stranded DNA (ssDNA) is recognized by RecA, and RecA in turn forms filaments around the ssDNA; at the same time, RecA induces the autocleavage of the repressor LexA, enabling the damage repair genes to be transcribed and the repair of DNA damage. In a *lon*-deficient mutant, the damage likely would not be repaired as effectively as in the wild type, due to the reduced induction of genes involved in the SOS response. Interestingly, upon exposure to 30 s of UV light and 4× MIC of ciprofloxacin (representing lethal conditions), the *lon* gene itself is slightly downregulated. Thus, it can be hypothesized that the Lon protease plays a role in controlling the expression level of the SOS response genes, possibly to suppress potential lethality associated with overexpression of *recA* and other SOS response genes and especially to limit the induction of cell death mediated by the phage pyocin operon (3). We

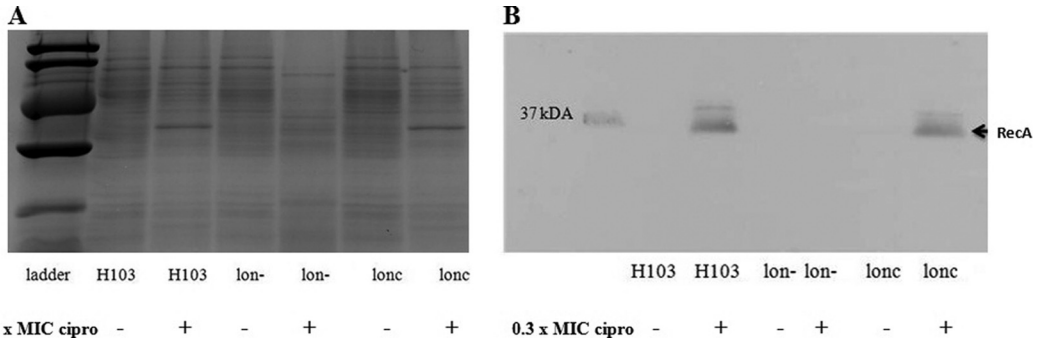

FIG 5 Induction of the *P. aeruginosa* RecA protein by subinhibitory concentrations of ciprofloxacin (cipro) in the wild type, the complemented *lon* strain, and the *lon* mutant. A Coomassie brilliant blue-stained SDS-PAGE gel (A) and an immunoblot of an identical gel are shown (B). Bacterial strains were grown overnight in the presence or absence of subinhibitory concentrations of ciprofloxacin, and whole-cell protein was loaded onto SDS-PAGE gels for analysis. For Western blotting, samples separated by SDS-PAGE were transferred to a PVDF membrane and incubated with a polyclonal antibody against RecA and a secondary antibody conjugated to HRP. Protein bands were developed with the ECL kit. Results from one of three repeats with similar data are shown.

were able to demonstrate that Lon does not act through *sulA* at the transcriptional level but likely works through SulA to regulate cell division. Therefore, we hypothesize that Lon acts through cleavage of one or more of the control elements of the SOS response. Since protein accumulation of RecA did not occur in the *lon* mutant upon ciprofloxacin exposure, RecA would not be able to cleave the repressor LexA to activate the SOS system. Furthermore, reduced RecA expression in the *lon* mutant would reduce binding to ssDNA, and thus, DNA damage could not be repaired. Lon is therefore important for modulating RecA function. It is also possible that the Lon protease might be important for cleaving other elements of the SOS response.

The ATP-dependent Lon protease investigated in this study controls the DNA stress response and fluoroquinolone susceptibility and is upregulated by aminoglycosides. *P. aeruginosa* has additional ATP-dependent proteases that exhibit similarity to PA1803. One of these proteases is the recently identified AsrA (aminoglycoside-induced stress response) ATP-dependent protease (PA0779), which is 60% similar to the Lon protease PA1803 (19). AsrA is highly upregulated by bacteriostatic and lethal concentrations of tobramycin and controls the heat shock stress response to tobramycin in *Pseudomonas*. It is thus becoming clear that ATP-dependent proteases are not only involved in general stress responses but have very specific roles and regulate and control important stress responses in *Pseudomonas*. The significant role of the Lon protease in virulence-related processes and antibiotic resistance makes it an attractive antimicrobial target to combat *P. aeruginosa* infections.

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