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Role of Lon, an ATP-Dependent Protease Homolog, in Resistance of *Pseudomonas aeruginosa* to Ciprofloxacin▽

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With few novel antimicrobials in the pharmaceutical pipeline, resistance to the current selection of antibiotics represents a significant therapeutic challenge. Microbial persistence in subinhibitory antibiotic environments has been proposed to contribute to the development of resistance. *Pseudomonas aeruginosa* cultures pretreated with subinhibitory concentrations of ciprofloxacin were found to exhibit an adaptive resistance phenotype when cultures were subsequently exposed to suprainhibitory ciprofloxacin concentrations. Microarray experiments revealed candidate genes involved in such adaptive resistance. Screening of 10,000 Tn*5*-*luxCDABE* mutants identified several mutants with increased or decreased ciprofloxacin susceptibilities, including mutants in PA1803, a close homolog of the ATP-dependent *lon* protease, which were found to exhibit ≥4-fold-increased susceptibilities to ciprofloxacin and other fluoroquinolones, but not to gentamicin or imipenem, as well as a characteristic elongated morphology. Complementation of the *lon* mutant restored wild-type antibiotic susceptibility and cell morphology. Expression of the *lon* mutant, as monitored through a luciferase reporter fusion, was found to increase over time in the presence of subinhibitory ciprofloxacin concentrations. The data are consistent with the hypothesis that the induction of Lon by ciprofloxacin is involved in adaptive resistance.

Fluoroquinolones are broad-spectrum antimicrobials used clinically to treat a range of gram-positive and gram-negative infections, including chronic *Pseudomonas aeruginosa* infections in cystic fibrosis (CF) patients (20). They target the bacterial enzymes DNA gyrase and topoisomerase IV, which are essential for maintenance of the appropriate DNA topological state for replication and transcription (17). As with other antimicrobials, however, extensive fluoroquinolone use and misuse have led to increasing quinolone resistance among bacterial pathogens. Resistance to fluoroquinolones by gram-negative pathogens such as *P. aeruginosa* is often mediated by mutations in the target enzymes and/or up-regulation of efflux pump expression, coupled with intrinsically low outer membrane permeability (20, 27). Quinolone exposure is also known to induce transient adaptive resistance responses, which are reversible upon removal of the antibiotic stressor (5, 20, 27). Moreover, fluoroquinolone resistance is often associated with cross-resistance to other quinolones as well as to nonquinolone agents (48), making the development of fluoroquinolone resistance a major clinical problem.

To combat the further development of resistance, a number of pharmacokinetic and pharmacodynamic parameters (e.g., mutant prevention concentrations) used to predict the emergence of fluoroquinolone resistance are being reevaluated and instituted (16, 51). In addition, more-comprehensive studies on the mechanisms of action of fluoroquinolones and other antimicrobials (3, 9, 13, 18, 43) are being pursued so as to better understand the interaction and responses of microbes to these antibiotics. We and others have studied transcriptional responses to ciprofloxacin exposure and demonstrated that even at subinhibitory concentrations, the expression of hundreds of genes is altered (9, 10, 44). It has been proposed that these gene expression changes involve adaptive responses to the inhibition of the antibiotic target as well as resistance mechanisms induced in an attempt by the bacterium to evade the lethal action of the antibiotic (10).

These functional genomic studies have also indicated that SOS stress responses can be evoked by fluoroquinolones (and other agents acting on replication mechanisms), even at subinhibitory concentrations (9, 44). Triggering of this response can subsequently result in an increased genome-wide mutation rate, enhancing the possibility of mutations in resistance genes. Subinhibitory concentrations of antibiotics are also being recognized for their role in microbial persistence and the development of antimicrobial resistance (4, 23). As such, subinhibitory concentrations of antibiotics represent a therapeutically important selective environment. This study utilized high-throughput genomic approaches to characterize the effects of ciprofloxacin on *P. aeruginosa* and to identify candidate genes important in mediating adaptive resistance to fluoroquinolones.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are described in the tables. *P. aeruginosa* PAO1 strain H103 was used as the wild type. In all experiments, overnight aerobic cultures were grown with agitation in Luria-Bertani (LB) broth (1.0% tryptone, 0.5% yeast extract, 5% NaCl; Difco Laboratories, Detroit, MI) at 37°C and were then used to inoculate 50 ml of LB broth in 500-ml Erlenmeyer flasks. Cultures were either left untreated or treated with 0.1×, 0.3×, or 1× MIC (0.01 μg/ml, 0.03 μg/ml, or 0.1 μg/ml) ciprofloxacin and were then grown to the mid-logarithmic phase (optical density at 600 nm [OD_{600}], 0.5 to 0.6). Ciprofloxacin was obtained from Bayer (United Kingdom), and solutions were made fresh daily.

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Adaptive resistance assay. The ability of ciprofloxacin to induce adaptive resistance was evaluated using a modified time-kill assay. Briefly, *P. aeruginosa* PAO1 strain H103 was grown to mid-logarithmic phase (OD_{600}, 0.5 to 0.6) in the presence or absence of ciprofloxacin (0.1× or 0.3× MIC). At mid-logarithmic phase, cells were pelleted by centrifugation. Supernatants were discarded and pellets washed in 10 ml of phosphate-buffered saline (pH 7.4; Invitrogen Co.) that had been prewarmed to 37°C. Cells were again pelleted by centrifugation and the supernatant decanted. Pellets were resuspended in 50 ml LB broth (prewarmed to 37°C) containing ciprofloxacin at 2× MIC (0.2 μg/ml) and were transferred to new 500-ml Erlenmeyer flasks for incubation with shaking at 37°C. Samples were taken every 15 to 20 min for 2 h and were plated at various dilutions onto LB agar plates for colony counts. Plates were incubated at 37°C overnight and CFU counted the next day. All assays were repeated in four independent experiments, and representative results are presented.

MIC determinations. The MICs of antibiotics against the various *P. aeruginosa* strains were measured using the broth microdilution technique (2). Antimicrobials were made fresh daily. Norfloxacin and nalidixic acid were obtained from Sigma, imipenem from Merck, and gentamicin from ICN Biomedicals Inc. (Aurora, OH). Growth was scored after 24 h of incubation at 37°C. Assays were repeated in quadruplicate, and the modes are presented.

Mutant library resistance and susceptibility screening. A *P. aeruginosa* mini-Tn*5-luxCDABE* mutant library (30) was screened for altered ciprofloxacin susceptibility. Bacteria were grown overnight as described above in 96-well plates containing LB broth and were diluted 1:1,000 in LB broth as for MIC determinations. A 48-pin replicator was then used to replicate stamp diluted cultures onto plates containing LB agar alone or with ciprofloxacin at 0.2 μg/ml, 0.1 μg/ml, 0.05 μg/ml, 0.03 μg/ml, or 0.01 μg/ml. LB plates were scored for the absence of growth after a 24-h incubation period at 37°C. The MICs for more highly resistant and susceptible mutants were examined more precisely by the broth microdilution assay.

Light microscopy. Microscope slides were inoculated with a loopful of bacteria taken from adaptive resistance assay cultures containing either untreated strain H103 or strain H103 pretreated with either 0.01 μg/ml, 0.03 μg/ml, or 0.1 μg/ml ciprofloxacin. Samples were taken before and after the phosphate-buffered saline wash and every 15 min thereafter throughout the assay exposure to 0.2 μg/ml ciprofloxacin. Samples were also taken from cultures of H1105 (*lon*::*lux*) and its complemented derivative H1105/*lon*$^+$ grown in the presence and absence of the respective 0.3× MIC ciprofloxacin concentrations. Cells were heat fixed, Gram stained, and examined at ×1,000 magnification. The lengths of 10 random cells were measured manually in micrometers.

Microarray processing and data analysis. Microarray studies were performed as previously described (9) and are described in outline below. All microarray experiments were performed on three independent cultures and were repeated at least twice per culture sample. RNA was isolated using QMIDI RNeasy columns (Qiagen, Inc.) and freed of contaminating DNA using a DNA-free kit (Ambion, Inc.). cDNA probes were generated with Superscript II reverse transcriptase using random primers annealed to total RNA (10 μg) and to five exogenous transcripts (ATCC 87482, ATCC 87483, ATCC 87484, ATCC 87485, and ATCC 87486) (130 pM) to incorporate amino-allyl dUTP (Ambion, Inc.). Residual RNA was removed by alkaline treatment followed by neutralization, and cDNA was salt-ethanol precipitated at −70°C. Precipitated cDNA was resuspended in 0.2 M $NaHCO_3$ (pH 9.0) and labeled with monoreactive Cy3/Cy5 dyes in dimethyl sulfoxide according to the manufacturer's instructions (Amersham Pharmacia).

Labeled sample pairs from mid-logarithmic-phase cultures that were either left untreated or treated with ciprofloxacin at 0.1×, 0.3×, or 1× MIC were combined and purified using a QIAquick PCR purification kit (Qiagen, Inc.), and the labeled cDNA sample was salt-ethanol precipitated and resuspended in hybridization buffer 2 (Ambion, Inc.). Custom in-house *P. aeruginosa* microarray slides (ArrayIT superamine slides) containing 400- to 600-bp PCR amplicons for 5,376 of the 5,570 *P. aeruginosa* genes were prepared for hybridization as described previously (9), hybridized with denatured labeled cDNAs, applied to a prepared microarray, overlaid with a coverslip, and hybridized overnight at 45°C in a hybridization chamber (Corning Inc.). Microarrays were scanned on a ScanArray Express scanner (Perkin-Elmer), and images were analyzed using ImaGene (version 5.0; BioDiscovery, Inc.). Background correction and normalization were performed using R program scripts (28). All genes in the ciprofloxacin treatment groups were compared to those in the untreated group using two sample *t* statistics and permutation *P* values to determine statistical significance, as well as GeneSpring (version 5.5; Silicon Genetics). Genes exhibiting permutation *P* values of ≤0.05 were considered differentially expressed.

Luminescence assay. Changes in luminescence as indicators of gene expression changes were analyzed by measuring luminescence from H1105 as described previously (30). Luminescence was monitored over time using a SPECTRAFluorPlus luminometer (Tecan, San Jose, CA). Cells were grown overnight and diluted 1:1,000 in fresh LB broth alone or supplemented with 0.3× MIC ciprofloxacin (0.0075 μg/ml). Triplicate samples from each culture were evaluated. Luminescence was corrected for growth by simultaneously monitoring the absorbance at 620 nm. The assay was repeated in triplicate.

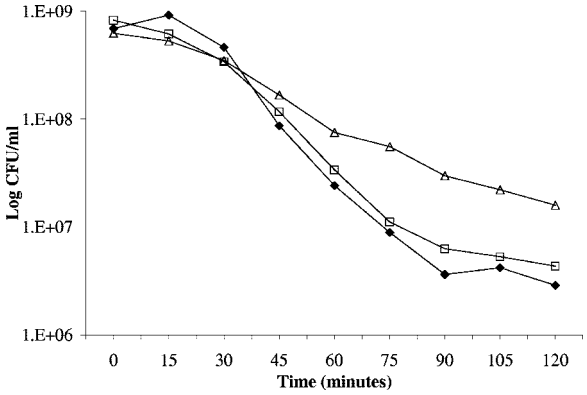

FIG. 1. Survival ability of *P. aeruginosa* in ciprofloxacin at 2× MIC following growth in subinhibitory concentrations of ciprofloxacin. Cultures of *P. aeruginosa* strain H103 were grown in the presence (□, 0.01 μg/ml; △, 0.03 μg/ml) or absence (◆) of subinhibitory concentrations of ciprofloxacin to mid-logarithmic phase; then they were washed and exposed to 0.2 μg/ml ciprofloxacin. CFU were counted every 15 min for 2 h. Results from a representative data set of four independent experiments are shown.

RESULTS

Subinhibitory ciprofloxacin concentrations induce adaptive resistance. To determine whether subinhibitory ciprofloxacin concentrations contribute to microbial persistence and the development of resistance, the survival in 2× MIC ciprofloxacin of *P. aeruginosa* cultures pretreated with subinhibitory ciprofloxacin concentrations was determined. Cultures pretreated with 0.03 μg/ml ciprofloxacin (0.3× MIC, based on a MIC of 0.1 μg/ml) consistently exhibited enhanced survival by approximately 1 log unit when exposed to 0.2 μg/ml ciprofloxacin (2× MIC); pretreatment with 0.1× MIC ciprofloxacin did not elicit appreciable survival advantages (Fig. 1). These results indicate the ability of this ciprofloxacin concentration to elicit adaptive resistance mechanisms in *P. aeruginosa*. Similar findings have been reported for cultures pretreated with 0.5× MIC and then exposed to 100× MIC ciprofloxacin (25).

Since ciprofloxacin, like other DNA-damaging agents, is a known inducer of SOS-mediated DNA repair mechanisms and cell filamentation (22, 40, 50), ciprofloxacin-exposed cultures were Gram stained and examined under a light microscope to discern the extent of cell division inhibition and whether this inhibition could have contributed to the adaptive resistance assay observations. Inhibition of cell division would result in an underestimation of the extent of survival, since a cell filament of 2 to 4 cell lengths would be recorded as a single surviving cell rather than two to four independent survivors. Examination of cultures immediately prior to exposure to 0.2 μg/ml ciprofloxacin revealed that *P. aeruginosa* cultures pretreated with inhibitory ciprofloxacin concentrations were moderately elongated (cell lengths, 3.0, 3.6, and 6.5 μm for 0.1×, 0.3×, and 1× MIC ciprofloxacin) relative to untreated cultures (2.8 μm)

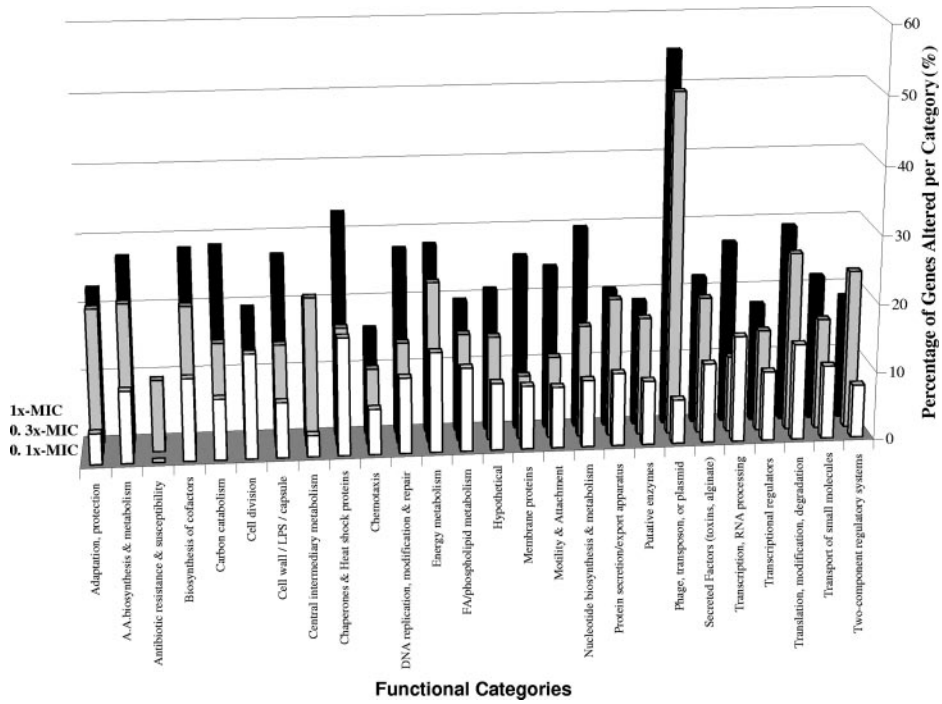

FIG. 2. Percentage of genes in each of the gene functional categories exhibiting expression changes after growth of *P. aeruginosa* in subinhibitory and inhibitory concentrations of ciprofloxacin. A.A., amino acid; FA, fatty acid.

(data not shown). Examination of cultures 1.5 h following exposure to 0.2 μg/ml ciprofloxacin found that cultures were elongated in a manner proportional to the initial pretreatment elongation, i.e., 4.8, 5.4, and 7.0 μm for untreated cultures and cultures pretreated with ciprofloxacin at 0.1× and 0.3× MIC, respectively (data not shown). Ciprofloxacin-pretreated cultures were not mucoid in appearance, an observation consistent with previous studies examining the effects of subinhibitory ciprofloxacin concentrations on mucoidy (44).

Subinhibitory ciprofloxacin concentrations alter the expression of many chaperone and heat shock proteins. Previously, DNA microarray technology was used to globally investigate the influence of subinhibitory (0.03 μg/ml [0.3× MIC]) and inhibitory (0.1 μg/ml [1× MIC]) concentrations of ciprofloxacin on the transcriptome of *P. aeruginosa*, demonstrating the induction of a phage-pyocin operon that determined susceptibility to ciprofloxacin at the MIC (9). These studies were extended here by the addition of microarrays for cultures treated with 0.1× MIC (0.01 μg/ml), and the combined data were analyzed to discern which open reading frames (ORFs) were likely contributors to the adaptive response of *P. aeruginosa* to subinhibitory ciprofloxacin concentrations.

Analysis of the microarray data with respect to functional classification found that 17% of the ORFs in the "Chaperones & Heat shock proteins" category were altered by subinhibitory ciprofloxacin concentrations (0.1× and 0.3× MIC) (Fig. 2). This percentage increased to 33% for cultures exposed to 1× MIC ciprofloxacin, highlighting SOS responses in the reaction of *P. aeruginosa* even to subinhibitory ciprofloxacin concentrations. ORFs categorized as related to a "Phage, transposon, or plasmid" were also highly affected by subinhibitory ciprofloxacin concentrations (Fig. 2) and previously led to the discovery of a fluoroquinolone susceptibility determinant (9). These observations are consistent with the known DNA replication interference and mutagenic activity of ciprofloxacin, activity that elicits an SOS DNA repair response (32, 39). Another functional class more strongly induced by ciprofloxacin at 0.3× MIC (26%) than at 0.1× MIC (12%) was the "Translation, modification, degradation" category (Fig. 2). PA1803, a close homolog (84% similarity) of the *Escherichia coli* Lon protease, is a member of this class.

Screening of a transposon library for mutants with altered susceptibilities. To further investigate the ORFs within the *P. aeruginosa* genome that are important in the response to ciprofloxacin, a mini-Tn*5-luxCDABE* mutant library (30) was screened for mutants exhibiting either increased susceptibility or increased resistance to ciprofloxacin, since mutation of a potential resistance ORF would result in increased susceptibility and mutation of a potential resistance regulatory ORF would elicit increased resistance. Mutants were replica plated onto various concentrations of ciprofloxacin. Of approximately 10,000 mutants, a total of 81 and 23 mutants, respectively, were found to display a fourfold decrease or twofold increase in the ciprofloxacin MIC on the plate assay. Of these mutants, 35 were sequenced and their insertion positions mapped in the *P. aeruginosa* genome (Tables 1 and 2). Striking among the ciprofloxacin-supersusceptible mutants was the number of mutants with alterations in PA1803 (*lon*), a close homolog of a DNA heat shock protein important in degrading SulA, an inhibitor of cell separation in *E. coli* (42). Other noteworthy supersusceptible mutants included those with alterations in penicillin binding protein 1B and various components of the DNA replication and repair machinery (Table 1), including *mrcB*, *sbcB*, *xseA*, *recA*, *recN*, and *recG*, all of which exhibited

TABLE 1. Sequenced and mapped *P. aeruginosa luxCDABE* mutant strains exhibiting at least twofold-increased ciprofloxacin susceptibility[a]

Strain	Allele	ORF	Gene name	Description
H1101	15_H4	PA0353	*ilvD*	Dihydroxyacid dehydrogenase
H1102	74_G10	PA0836		Probable acetate kinase
H1104	31_C9	PA1803	*lon*	Lon protease
H1105	74_D9	PA1803	*lon*	Lon protease
H1106	24_A2	PA1803	*lon*	Lon protease
H1107	50_F11	PA3082	*gbt*	Glycinebetaine transmethylase
H1108	73_E4	PA3294		Hypothetical protein
H1109	69_C12	PA3617	*recA*	DNA recombinase RecA
H1110	76_E9	PA3777	*xseA*	Exodeoxyribonuclease VII large subunit
H1111	14_E7	PA3965		Probable transcriptional regulator
H1112	51_E8	PA4163		Hypothetical protein
H1113	82_F7	PA4316	*sbcB*	Exodeoxyribonuclease I
H1114	98_C2	PA4316	*sbcB*	Exodeoxyribonuclease I
H1115	81_B12	PA4422		Conserved hypothetical protein
H1116	43_C12	PA4466		Probable phosphoryl carrier protein
H1117	22_C1	PA4700	*mrcB*	Penicillin binding protein 1B
H1118	73_D6	PA4700	*mrcB*	Penicillin binding protein 1B
H1119	75_D3	PA4756	*carB*	Carbamoylphosphate synthetase large subunit
H1120	81_D2	PA4763	*recN*	RecN DNA repair protein
H1121	6_G1	PA4961		Hypothetical protein
H1122	80_B7	PA5016	*aceF*	Dihydrolipoamide acetyltransferase
H1123	22_C5	PA5208		Hypothetical protein
H1124	53_F11	PA5279		Hypothetical protein
H1125	16_G12	PA5345	*recG*	ATP-dependent DNA helicase RecG

[a] As assayed by an antibiotic plate screen. Mutant alleles in our Tn*5-lux* library are described online at http://pseudomutant.pseudomonas.com/ and in reference 30.

increased expression following ciprofloxacin exposure, as assayed by microarrays (9). Prominent among the ciprofloxacin-resistant mutants were those with mutations of *nfxB*, the known transcriptional regulator for MexCD-OprJ, one of the major multidrug efflux proteins contributing to fluoroquinolone resistance (Table 1) (41), and *rnt*, the altered expression of which was also identified by microarray analysis (9).

MICs for a number of the supersusceptible and resistant mutants identified by the plate assay were confirmed by the broth microdilution method (Table 3). Among the confirmed mutants, all displayed consistent changes in susceptibilities to ciprofloxacin, norfloxacin, and naladixic acid but not to other antibiotics (Table 3). Subsequent studies were directed to further characterization of Lon.

Fluoroquinolone MICs are lower for *lon* mutants. Among the confirmed supersusceptible mutants, ciprofloxacin MICs for *lon* mutants were found to be fourfold decreased from that for wild-type *P. aeruginosa* (Table 3). *lon* mutants were also found to be at least twofold more sensitive to norfloxacin and twofold more sensitive to nalidixic acid (Table 3). Slight differences in the MICs of fluoroquinolones for the various *lon* mutants correspond with differences in their insertion points, whereby H1104 and H1106 have Tn*5-lux* inserts at nucleotides 1958047 and 1958055, respectively, more than 450 nucleotides downstream from the insertion site for H1105 at nucleotide 1957592 (30). The imipenem and gentamicin MICs were unchanged, and fluoroquinolone MICs were restored to wild-type levels by complementation of the H1105 (*lon*::*lux*) mutant (Table 3), suggesting that the hypersusceptibility observed following the disruption of *lon* was specific to fluoroquinolones. However, expression of the *lon* gene, as assessed by luminescence of the *lon*::*lux* (H1105) transcriptional fusion, increased in cultures grown in subinhibitory ciprofloxacin concentrations (0.3× MIC; 0.0075 μg/ml) relative to expression in cultures grown without ciprofloxacin (Fig. 3).

The importance of *lon* in eliciting adaptive resistance was reexamined for H1105 (*lon*::*lux*) in the adaptive resistance assay. When the appropriate strain-specific ciprofloxacin concen-

TABLE 2. Sequenced and mapped *P. aeruginosa luxCDABE* mutant strains exhibiting at least twofold-increased ciprofloxacin resistance[a]

Strain	Allele	ORF	Gene name	Description
H1126	51_C1	PA0393	*proC*	Pyrroline-5-carboxylate reductase
H1127	75_F2	PA0413		Probable chemotactic signal transduction system
H1128	41_C11	PA3115	*fimV*	Motility protein FimV
H1129	70_H3	PA3528	*rnt*	RNase T
H1130	51_E1	PA3761		Probable phosphotransferase system protein
H1131	50_D4	PA3823	*tgt*	Queuine tRNA-ribosyltransferase
H1132	50_D5	PA4556	*pilE*	Type IV fimbrial biogenesis protein PilE
H1133	63_H5	PA4600	*nfxB*	Transcriptional regulator NfxB
H1134	50_C11	PA4695	*ilvH*	Acetolactate synthase isozyme III subunit
H1135	47_B5	PA5117	*typA*	Regulatory protein TypA
H1136	75_F11	PA5455		Hypothetical protein

[a] As assayed by an antibiotic plate screen. Mutant alleles in our Tn*5-lux* library are described online at http://pseudomutant.pseudomonas.com/ and in reference 30.

TABLE 3. MICs of different antimicrobials for various strains of *P. aeruginosa* grown in LB medium

Strain	Allele[a]	Mutation	MIC (μg/ml)[b] of:				
			CIP	NOR	NAL	IMI	GM
Sensitive strains							
H103		Wild type	0.1	0.3	256	1	2
H1104	31_C9	*lon*::*lux*	0.025	0.15	128	1	2
H1105	74_D9	*lon*::*lux*	0.025	0.075	128	1	2
H1106	24_A2	*lon*::*lux*	0.025	0.15	128	1	2
H1109	69_C12	*recA*::*lux*	0.05	0.075	256	1	2
H1121	6_G1	PA4961::*lux*	0.05	0.075	128	1	2
H1124	53_F11	PA5279::*lux*	0.05	0.15	128	1	2
H1125	16_G12	*recG*::*lux*	0.025	0.15	128	1	2
H1105/*lon*$^{+}$[c]		*lon*::*lux*/*lon*$^{+}$	0.1	0.3	256	1	—[d]
Resistant strains							
H103		Wild type	0.1	0.3	256	1	2
H1129	70_H3	*rnt*::*lux*	0.4	>0.6	512	1	2
H1133	63_H5	*nfxB*::*lux*	0.8	>0.6	512	1	2
H1136	75_F11	PA5455::*lux*	0.4	>0.6	512	1	2

[a] Mutant alleles in our Tn*5*-*lux* library are described online at http://pseudomutant.pseudomonas.com/ and in reference 30.
[b] Results are modes from four independent experiments. CIP, ciprofloxacin; NOR, norfloxacin; NAL, nalidixic acid; IMI, imipenem; GM, gentamicin.
[c] H1105 (*lon*::*lux*) complemented with the Lon-expressing plasmid pBBR1-MCS5::lon^{+} (33).
[d] Not measurable, because this resistance marker was on the cloning plasmid.

trations (0.3× MIC; 0.03 μg/ml for H103 and 0.0075 μg/ml for H1105) were used, no survival of H1105 cultures in supra-inhibitory ciprofloxacin concentrations was observed, in contrast to the results in Fig. 1 for ciprofloxacin-pretreated wild-type cultures. Microscopic analysis of cell lengths for the H1105 mutant grown in the absence or presence of ciprofloxacin at 0.3× MIC showed that these H1105 cells were exceptionally elongated (Fig. 4). In *E. coli*, the hypersusceptibility of *lon* mutants is due both to fluoroquinolone induction of the cell separation inhibitor SulA and the subsequent accumulation of SulA, which contributes to quinolone killing by inhibiting cell division (49). Similar mechanisms likely contribute to the diminished capacity of *P. aeruginosa lon* mutants to survive ciprofloxacin exposure, given the elongated cell morphology of such mutants. Complementation of the *lon* mutant (H1105/*lon*$^{+}$) was found to restore cell division, as assessed on the basis of cell morphology (Fig. 4).

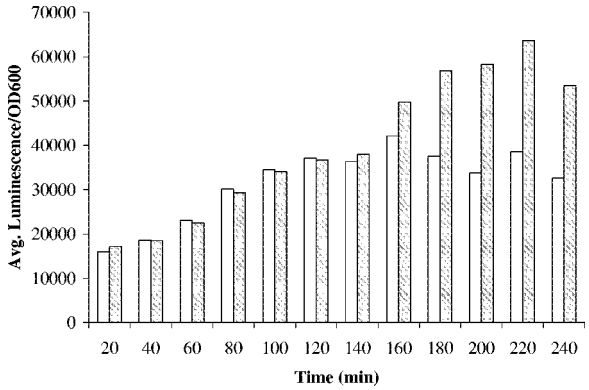

FIG. 3. Induction of the *lon*::*luxCDABE* fusion (H1105) with 0.3× MIC ciprofloxacin (shaded bars) over expression in untreated H1105 cultures (open bars). Results are modes from four independent experiments.

DISCUSSION

Accumulation of single-stranded DNA regions at transcription or replication forks following DNA-damaging events stalls the polymerase machinery and necessitates repair of the lesion if the DNA molecule is to continue to serve as the template for transcription or replication. The SOS system functions mainly in the repair of such DNA damage. However, the SOS response to DNA damage has also recently been shown to promote the horizontal dissemination of antibiotic resistance genes (6). Others have similarly shown induction of prophages in *E. coli* (29, 45), *Staphylococcus aureus* (21), and *P. aeruginosa*

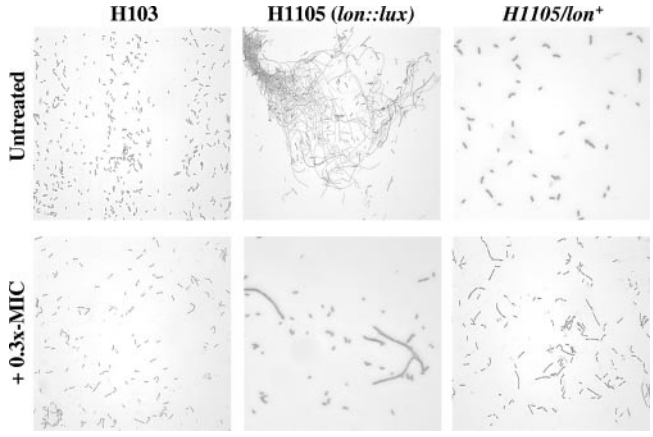

FIG. 4. Gram-stained *P. aeruginosa* strains grown for 3 h in the presence or absence of subinhibitory ciprofloxacin concentrations. As evident from the Gram stain images of cells grown in the absence or presence of 0.3× MIC ciprofloxacin [0.03 μg/ml for H103 and H1105/*lon*$^{+}$; 0.0075 μg/ml for H1105 (*lon*::*lux*)], cell division of *P. aeruginosa* mutants with alterations in the Lon protease homolog [H1105 (*lon*::*lux*)] was inhibited relative to that of wild-type H103 cells. Complementation of the *lon* mutant (H1105/*lon*$^{+}$) restored cell division. All micrographs are at ×1,000 magnification.

(9), likewise implying that stress conditions such as antibiotic treatment can promote lateral gene transfer through induction of the SOS system, thereby accelerating the acquisition and spread of antibiotic resistance. These findings have important implications for the use of antibiotics, because the nonlethal use of many antibiotics, particularly DNA-damaging agents such as ciprofloxacin, can induce the SOS response and potentially enhance the transmission of resistance (10, 26). Of even greater concern is recent evidence demonstrating the capacity of sublethal concentrations of fluoroquinolones to also mediate SOS-independent recombination (31). These findings further highlight our naïve comprehension of the full spectrum of antibiotic-microbe interactions and effects.

To address these deficiencies, recent antimicrobial research has focused on induction of the SOS response by antibiotic environments and the role that SOS responses play in resistance development. In addition to the well-characterized role of inhibitory concentrations, subinhibitory concentrations of antibiotics may also be critical in this context. Sublethal concentrations are particularly concerning because, as Baquero (4) argues, poor compliance, poor absorption, selective compartments, and biofilms, among other factors, all contribute to engendering suboptimal antibiotic concentrations. We have expanded on findings about the effects of inhibitory concentrations of antibiotics by examining the response of *P. aeruginosa* to subinhibitory concentrations of the DNA gyrase inhibitor ciprofloxacin and the role such nonlethal concentrations play in inducing the SOS response and antibiotic resistance. Ciprofloxacin at 0.3× MIC was found to enhance the ability of pretreated cultures to survive under 2× MIC ciprofloxacin conditions (Fig. 1). Such a survival phenomenon has been noted in many bacterial species and has been termed adaptive resistance (which may be related to persistence), since the fraction of cells that survive antibiotic challenge have not acquired antibiotic resistance through genetic mutation or resistance gene acquisition. Rather, the fraction of cells that survive have the capacity to switch on a mechanism that allows the cell to survive antibiotic challenge, without the concomitant fitness cost of a genetically encoded resistance mechanism (34, 35). Similar phenotypic tolerance or adaptive resistance findings have been made previously for inhibitory concentrations of ciprofloxacin (24, 47) and aminoglycosides (5, 14). Both subinhibitory and inhibitory concentrations of ciprofloxacin, therefore, have the ability to select for such resistance.

The mechanisms behind adaptive resistance, however, are poorly understood. Drenkard and Ausubel reported that antibiotic treatment of *P. aeruginosa* CF infections selects for a transient resistance phenotype and that the regulator PvrR is important in eliciting this phenotypic variation (since overexpression of PvrR results in a reduction in the frequency of resistant variants) (15). In our previous studies, microarray analysis was used to examine the effects of subinhibitory ciprofloxacin concentrations on global gene expression in *P. aeruginosa* (9), and this was expanded here using 0.1× MIC ciprofloxacin concentrations. Interestingly, the expression of *pvrR* (PA3947) was down-regulated in 0.3× MIC ciprofloxacin, consistent with the suggestion of Drenkard and Ausubel (15) that lack of *pvrR* expression may contribute to the observed adaptive resistance phenotype.

Other mechanisms related to intrinsic resistance may also result in adaptive resistance. Walsh and colleagues demonstrated that plasmid expression of the *P. aeruginosa* lipopolysaccharide kinase genes *waaP* and *wapP* in a *Salmonella enterica* serovar Typhimurium strain carrying a chromosomal mutation in *waaP* resulted in a substantial increase in resistance to the DNA gyrase inhibitor novobiocin (46). These genes are important in phosphorylation of the inner core of *P. aeruginosa* lipopolysaccharide, and thus they are important in contributing to the intrinsic drug resistance of this organism. Expression levels of both *waaP* (PA5009) and *wapP* (PA5008) were significantly increased with ciprofloxacin concentrations of 0.3× MIC (1.4- and 1.5-fold, respectively) and 1× MIC (1.8- and 1.9-fold, respectively) (9), demonstrating that adaptive resistance to subinhibitory ciprofloxacin concentrations may also result from enhanced intrinsic resistance mechanisms.

Subinhibitory ciprofloxacin concentrations were also found to activate the SOS system. Microarray studies showed that expression of *recA*, the principal regulator of the SOS response, was significantly up-regulated with increasing ciprofloxacin concentrations (9). Analysis of the expression profiles based on functional classification of the ORFs further supported this observation (Fig. 2). The effects of both 0.1× and 0.3× MIC ciprofloxacin concentrations on ORFs belonging to the "Chaperones and heat shock proteins" category again emphasize the importance of the SOS system in the response of *P. aeruginosa* to even sublethal antibiotic challenge. These findings highlight the importance of even sublethal concentrations of antibiotics, particularly ciprofloxacin, in provoking the SOS response (although stimulation of intrachromosomal recombination by subinhibitory levels of ciprofloxacin in *E. coli* has also been shown to occur independently of this response [31]). This SOS response is already known to promote horizontal gene transfer (6) and increased mutation rates (7, 34, 36), both of which are possible mechanisms by which antibiotic resistance can be acquired. Indeed, Fung-Tomc et al. (19) noted an increase in the mutation rate and resistance level of *P. aeruginosa* following exposure to subinhibitory ciprofloxacin concentrations, whereby the rate of resistance development depended on the concentration and the duration of exposure. Prolonged exposure to subinhibitory ciprofloxacin concentrations was also found to promote the development of low-level resistance to structurally unrelated antimicrobial agents (19).

Because the microarray studies, therefore, seemed to suggest that a combination of mechanisms, rather than a solitary mechanism, mediated adaptive resistance, a mini-Tn*5-luxCDABE* mutant library (30) was screened in order to better characterize some of the factors responsible for resistance to ciprofloxacin. Many of the mutants identified as exhibiting increased sensitivity to ciprofloxacin (Table 1) had alterations in genes belonging to the "DNA replication, modification, and repair" class as well as being components of the SOS DNA repair system. For example, *xseA*, along with *xseB*, encodes exonuclease VII, which degrades single-stranded DNA (11). The ciprofloxacin hypersusceptibility of an *xseA* mutant correlates well with the ability of fluoroquinolone antimicrobials to produce DNA breaks (17). Likewise, the ciprofloxacin hypersusceptibility of a *recN* mutant agrees well with its role as an SOS-inducible protein important in repairing double-stranded DNA breaks (38). Conversely, resistance to ciprofloxacin was also observed to increase (Table 2) for mutants with alterations in

nfxB, the negative regulator of the MexCD-OprJ efflux pump, which is known to be involved in fluoroquinolone resistance (41).

Of interest in determining the mechanisms responsible for the adaptive resistance response to ciprofloxacin were the supersusceptible mutants identified. Among these mutants, *lon* mutants were found to be fourfold more susceptible to ciprofloxacin than the wild-type strain (Table 3). Lon protease is a DNA heat shock protein important in cell separation (42) and, interestingly, is classified under "Translation, modification, degradation," the category of ORFs highly affected by subinhibitory ciprofloxacin concentrations (Fig. 2). Our observation of a phenotype of hypersusceptibility to fluoroquinolones for the *lon* mutants was in agreement with previous findings for *E. coli* (49). No similar hypersusceptibility phenotypes were observed for imipenem or gentamicin (Table 3), although a moderate decrease in the gentamicin MIC was previously noted (33). Although evaluation of *lon* overexpression would confirm its role in ciprofloxacin resistance, previous studies have shown genetic overexpression of *lon* to be lethal (12). Expression of *lon* was thus monitored from its luciferase transcriptional fusion and was found to increase when bacteria were grown in subinhibitory ciprofloxacin concentrations (Fig. 3), indicating that *lon* plays a role in the response of *P. aeruginosa* to ciprofloxacin exposure. Interestingly, Marr et al. (33) have shown that the expression of *lon* increases upon exposure to aminoglycosides at sub-MIC concentrations and that the mutant is defective in biofilm formation and swarming motility, findings that indicate that Lon protease may play a larger role in the pathogenesis of *Pseudomonas*.

Based on the extreme filamentation observed for *lon* mutants grown alone or in the presence of subinhibitory ciprofloxacin concentrations (Fig. 4) and on the wild-type susceptibility of *lon* mutants to other antimicrobials (Table 3), it is hypothesized that increased fluoroquinolone sensitivity is due to abnormal cell elongation. Indeed, Yamaguchi et al. demonstrated the suppression of *lon* hypersusceptibility in *E. coli* by subsequent disruption of *sulA*, the cell division inhibitor (49). If cell elongation is a contributing mechanism in fluoroquinolone killing, this would explain the inability to repeat the adaptive resistance phenotype (Fig. 1) with the *lon* mutants, since exposure to suprainhibitory ciprofloxacin concentrations would occur after cells had already achieved a filamentous state. Two *sulA* homologs (PA0671 and PA3008) in *P. aeruginosa* have been described (1). Of these, only PA3008, which lies adjacent to the SOS response transcriptional repressor LexA, showed increases in expression by microarray analysis (2.8-, 5.2-, and 6.5-fold increases for 0.1×, 0.3×, and 1× MIC ciprofloxacin, respectively) across all ciprofloxacin concentrations (9). Together, these findings indicate that PA1803 is likely to be the Lon ATP-dependent protease and that it might be a potential therapeutic antimicrobial target to combat fluoroquinolone resistance in *P. aeruginosa*.

Subinhibitory concentrations of antimicrobials, particularly of SOS response-inducing agents such as ciprofloxacin, thus play an important role in eliciting antibiotic resistance. Recent work by Blazquez et al. similarly showed that exposure of *P. aeruginosa* to subinhibitory concentrations of ceftazidime, a PBP3 inhibitor, also elicits an SOS response, resulting in increased mutagenesis and up-regulation of antibiotic resistance and adaptation genes (8). Interestingly, ceftazidime was also found to antagonize the activity of ciprofloxacin (8), a finding that bears particular relevance to the treatment regimens of CF patients. Subinhibitory concentrations of antimicrobial peptides have also been found to induce the *pmrAB* two-component regulator, leading to adaptive resistance (37). As our knowledge of the effects of subinhibitory and inhibitory concentrations of antibiotics on bacterial responses continues to grow, it is becoming increasingly evident that this knowledge should be considered in choosing antibiotic therapies and dosing regimens.

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