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Ciprofloxacin Induction of a Susceptibility Determinant in *Pseudomonas aeruginosa*†

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With few novel antimicrobials in development, resistance to the current selection of antibiotics increasingly encroaches on our ability to control microbial infections. One limitation in our understanding of the basis of the constraints on current therapies is our poor understanding of antibiotic interactions with bacteria on a global scale. Custom DNA microarrays were used to characterize the response of *Pseudomonas aeruginosa* to ciprofloxacin, a fluoroquinolone commonly used in therapy against chronic infections by this intrinsically resistant bacterium. Of the approximately 5,300 open reading frames (ORFs) on the array, 941 genes showed statistically significant ($P \leq 0.05$) differential expression in response to $0.3\times$ MIC of ciprofloxacin; 554 were promoted and 387 were repressed. Most striking among the responsive genes was the region between PA0613 and PA0648, which codes for the bacteriophage-like R2/F2 pyocins. In this region, virtually every ORF was increased by $0.3\times$ MIC of ciprofloxacin and even more dramatically up-regulated (7- to 19-fold) following treatment with $1\times$ MIC of ciprofloxacin. Pyocin gene expression was confirmed with *lux* reporter mutants and real-time PCR studies; pyocin-like particles were also present in transmission electron micrographs of supernatants from cells treated with $1\times$ MIC of ciprofloxacin. Interestingly, mutants in this region exhibited $\geq$8-fold-increased resistance to ciprofloxacin and other fluoroquinolones, demonstrating that this region is a susceptibility determinant. Since this region is known to be variably present in the genomes of clinical isolates of *P. aeruginosa* (R. K. Ernst et al., Environ. Microbiol. 5:1341–1349, 2003, and M. C. Wolfgang et al., Proc. Natl. Acad. Sci. USA 100:8484–8489, 2003), these findings demonstrate that the R2/F2 pyocin region is a "loaded gun" that can mediate fluoroquinolone susceptibility in *P. aeruginosa*.

Of particular concern in clinical settings is the development of "superbugs" which have acquired resistance to the vast majority of available antimicrobials, severely limiting treatment options and thus clinical outcomes. *Pseudomonas aeruginosa* is an important opportunistic pathogen because of its high intrinsic resistance to most antimicrobials used in therapeutic practice. This high intrinsic resistance is primarily due to a low outer membrane permeability coupled with various secondary resistance mechanisms (9), which effectively take advantage of the low antibiotic exposure afforded by the low permeability. Antibiotic exposure is further slowed by the propensity of the organism to become mucoid (23) and adopt a biofilm growth mode (2, 3), thereby permitting rapid mutation of the organism to a clinically relevant resistance state (8) and earning *Pseudomonas* "superbug" status.

In the absence of novel antimicrobials, an ability to control the development of antibiotic resistance to the current repertoire of antibiotics is critical. However, we are limited by our lack of knowledge of the interactions with pathogens and mechanisms of action of antimicrobial agents. This lack of knowledge constrains current therapies and consequently the development of new antibiotics. In an attempt to better under-

stand these factors, we utilized DNA microarray technology to investigate the influence of subinhibitory and inhibitory concentrations of ciprofloxacin on the transcriptome of *P. aeruginosa*. Similar microarray studies on the interactions of antibiotics with other medically important bacteria have defined expression patterns or "antimicrobial signatures" for these agents that in part reflect the mechanism of action of the antimicrobial being examined, as well as the pathogen being studied (7, 14, 22). Ciprofloxacin interaction with *Haemophilus influenzae* at inhibitory and suprainhibitory conditions, for example, was reported to mainly induce the expression of SOS and DNA repair systems, an expression pattern reflective of the organism's attempt to protect itself from the DNA damage effects of DNA gyrase inhibition (7). Analysis of antimicrobial signatures, however, can become complicated by the inhibition of secondary targets, as well as downstream effects that result from inhibition of the primary target (26); subinhibitory concentrations of ciprofloxacin were therefore used to reduce such complicating effects.

Ciprofloxacin remains a clinically relevant antimicrobial in the treatment of *P. aeruginosa* infections, particularly chronic cystic fibrosis infections (6), and is thought to be well characterized with respect to mechanism of action and resistance (12). In this study, we utilized a functional genomic approach to demonstrate that *P. aeruginosa* strain PAO1 has a previously unknown fluoroquinolone susceptibility determinant.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are described in Table 1. *P. aeruginosa* PAO1 strain H103 was used for

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TABLE 1. *Pseudomonas aeruginosa* strains used in this study

Strain	Genotype	Abbreviation	Reference or source
H103	Wild-type *P. aeruginosa* PAO1	H103	
57	PA0611::IS*lacZ*/hah derivative of PAO1	*prtR::lacZ*	15
6322	PA0613::IS*lacZ*/hah derivative of PAO1	PA0613::*lacZ*	15
PAO1_lux_22_E4	PA0620::*luxCDABE* derivative of H103; Tc^r	PA0620::*lux*	This study
3501	PA0621::IS*lacZ*/hah derivative of PAO1	PA0621::*lacZ*	15
PAO1_lux_26_H2	PA0641::*luxCDABE* derivative of H103; Tc^r	PA0641::*lux*	This study
43080	PA3617::IS*phoA*/hah derivative of PAO1	*recA::phoA*	15
PAO1_lux_24_A3	PA3866::*luxCDABE* derivative of H103; Tc^r	PA3866::*lux*	This study

most studies. 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 used to inoculate 50-ml portions of LB broth in 500-ml Erlenmeyer flasks. Cultures were either untreated or treated with 0.3× or 1× MIC of ciprofloxacin and grown to the mid-logarithmic phase (optical density at 600 nm, 0.5 to 0.6). All microarray experiments were performed on three independent cultures and repeated at least twice per culture sample. Ciprofloxacin hydrochloride was obtained from Bayer (United Kingdom) and made fresh daily in distilled water.

Determination of MICs. The MICs of antibiotics for the various *P. aeruginosa* strains were measured using the broth microdilution technique (1). Antimicrobials were made fresh daily and were obtained from the following sources: enofloxacin (Warner Lambert Co., Ann Arbor, MI), gentamicin (ICN Biomedicals Inc., Aurora, Ohio), norfloxacin (Sigma), nalidixic acid (Sigma), cefepime (Bristol Myers Squibb), and amikacin (Bristol Laboratories, Belleville, Ontario, Canada).

Microarray processing and data analysis. RNA was isolated using QMIDI RNeasy columns (QIAGEN, Inc.). Contaminating DNA was eliminated using a DNA-Free kit (Ambion, Inc.). cDNA probes were generated with random primers (NSNSNSNSNS) annealed to total RNA (10 µg) and to five exogenous transcripts (ATCC 87482, 87483, 87484, 87485, and 87486; 130 pM). Random primers were annealed at 70°C for 10 min and 25°C for 10 min, followed by 37°C for 60 min, 42°C for 120 min, and 70°C for 10 min. Reactions contained 1,500 U Superscript reverse transcriptase II (Invitrogen Co.), 1× Superscript reverse transcriptase II buffer, 3 mM amino-allyl dUTP (Ambion, Inc.), 3 mM dTTP, 10 mM (each) dATP, dCTP, and dGTP (Invitrogen Co.), and 30 U SUPERase-In (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 Cy3/5 dyes in dimethyl sulfoxide (Amersham Pharmacia; as per manufacturer's instructions).

Labeled sample pairs were then combined accordingly and purified with a QIAquick PCR purification kit (QIAGEN, Inc.). 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 cDNA probes (isolated as PCR amplicons) for 5,376 of the 5,570 *P. aeruginosa* genes were prepared for hybridization by washing two times for 5 min in 0.1% sodium dodecyl sulfate and five times for 1 min in water, boiling for 3 min in 95°C water, and air drying. Denatured sample was applied to a prepared microarray, overlaid with a Lifter-Slip coverslip (Electron Microscopy Sciences), and sealed in a hybridization chamber (Corning Inc.) for overnight hybridization at 45°C. Glass coverslips were removed by flotation in warm 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and slides were washed three times each for 5 min in 25 ml 0.5× SSC–0.5% sodium dodecyl sulfate and 25 ml 0.5× SSC and spun dry at 2,000 rpm for 5 min.

Microarrays were scanned on a ScanArray Express scanner (Perkin-Elmer) and images analyzed using ImaGene v5.0 (BioDiscovery, Inc.). The lowest 10 percent of signal for each subgrid was taken as background (R code developed by D. Hoffart and J. Brumm, Genome BC Array Facility). Output files were then compared and analyzed using R program scripts. All microarrays were normalized using the R package *vsn* (13). All genes in the 0.3× and 1× MIC of ciprofloxacin treatment group were compared to the untreated condition. To assess the statistical significance, two-sample *t* statistics and permutation *P* values were computed for each gene pair. Genes exhibiting permutation *P* values of ≤0.05 were considered differentially expressed. Data were also analyzed using GeneSpring v5.5 (Silicon Genetics) as a secondary analysis method. The changes (*n*-fold) presented herein are derived from GeneSpring v5.5 analysis and are shown only for those genes with statistically significant expression changes.

Microarray validation. Relative real-time PCR was performed on the ABI Prism 7000 sequence detection system using SYBR green I dye chemistry (Applied Biosystems). Primers were designed using sequence from the *Pseudomonas* database (www.pseudomonas.com) and the ABI Primer Express program v2.0 in the default mode and are listed in Table 2. De novo cDNA was made from 0.3× MIC of ciprofloxacin-treated strain H103 RNA used in microarray target preparation. Data were analyzed using the ABI Sequence Detection software (ABI).

Luminescence changes from PA0620::*lux* and PA0641::*lux* were monitored over time using a SPECTRAFluorPlus luminometer (Tecan, San Jose, CA). Cells were grown as described above in LB broth supplemented with 0, 0.01, 0.03, or 0.1 µg/ml ciprofloxacin, 0, 50, 150, or 500 µg/ml novobiocin (Sigma-Aldrich Corp.), or 0, 0.2, 0.6, or 2 µg/ml ceftazidime (GlaxoSmithKline Beecham, Inc.). Triplicate samples were removed hourly for luminescence analysis and measurement of growth at an optical density of 600 nm; averaged luminescence values were corrected for growth. The assay was repeated in triplicate.

Pyocin/phage isolation and transmission electron microscopy. Pyocin/phage-like particles were isolated and examined as previously described (29) from H103 and PA0620::*lux* cultures exposed to 1× the MIC of ciprofloxacin as described above for microarray experiments. Samples were placed on Formvar-coated grids, negatively stained with 1% phosphotungstate, and examined by transmission electron microscopy (Hitachi H7600 TEM) under standard operating conditions.

Microarray accession numbers. ArrayExpress accession numbers are A-FPMI-1 and E-FPMI-1.

RESULTS

The pyocin/phage region is up-regulated in response to subinhibitory ciprofloxacin. The MIC of ciprofloxacin for *P. aeruginosa* PAO1 strain H103 (0.1 µg/ml [Table 3]) was comparable to the MIC for other sensitive *Pseudomonas* strains (5) and was used to calculate the 0.3× the MIC subinhibitory concentration. The growth rate of strain H103 was not found to be appreciably affected by subinhibitory or inhibitory concentrations of ciprofloxacin during a 2.5- to 3-h exposure; growth

TABLE 2. Primer sequences used in this study

Primer	Sequence
PA0610_sense	5′-TAGCACTCCGATTCCACGC-3′
PA0610_antisense	5′-CCGAAGATGCGGTAGACCA-3′
PA0611_sense	5′-AGCTTCAACCGCGAGGAATA-3′
PA0611_antisense	5′-CATGTCCTCCGGCGAGTACT-3′
PA0621_sense	5′-TTTCCCGTCAGCAACGTAGC-3′
PA0621_antisense	5′-GCTGACTATCCCGCCATCTC-3′
PA0623_sense	5′-CCGAGAAGTCCCGTGAATTTCT-3′
PA0623_antisense	5′-CCATTGAAAGCGCTCTGGTC-3′
PA3617_sense	5′-GTGAAGAACAAGGTTTCCCCG-3′
PA3617_antisense	5′-GAGGATCTGGAACTCGGCCT-3′
PA3866_sense	5′-CCACTTGTCGTGACCAGAGGA-3′
PA3866_antisense	5′-CATCGACCCAGGCTCGTAA-3′
*rplF*_sense	5′-AGGTTGCTGCCGAAATTCG-3′
*rplF*_antisense	5′-CTTGCCTTTGTAAGGCTCCG-3′

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

Strain	MIC (μg/ml)[a]						
	CIP	ENO	NOR	NAL	AMK	FEP	GAT
H103	0.06	0.3	0.15	15.6	0.4	0.6	1.75
prtR::*lacZ*	0.06	0.3	0.15	31.25	0.4	0.6	0.875
PA0613::*lacZ*	0.5	1.6	>2.5	250	0.4	0.6	0.875
PA0620::*lux*	0.5	1.6	>2.5	250	0.4	1.25	0.875
PA0621::*lacZ*	0.5	>5	>2.5	250	0.2	0.3	0.875
PA0641::*lux*	0.5	1.6	1.25	125	0.4	1.25	1.75
recA::*phoA*	0.5	3.2	>2.5	250	0.4	0.6	0.875
PA3866::*lux*	0.06	0.3	0.15	31.25	0.4	0.3	ND

[a] Results shown are the mode of four independent experiments. CIP, ciprofloxacin; ENO, enofloxacin; NOR, norfloxacin; NAL, naldixic acid; AMK, amikacin; FEP, cefepime; GAENT, gentamicin; ND, not determined.

thereafter was severely limited by the MIC (Fig. 1). All subsequent experiments were conducted using cultures treated for 2.5 h.

DNA microarray technology was then 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*. Microarray analysis of *P. aeruginosa* strain H103 cells treated with 0.3× MIC of ciprofloxacin identified a total of 941 genes, approximately 15% of the known open reading frames (ORFs), as being significantly affected by the treatment ($P \leq 0.05$) (a complete list of genes is available in the supplemental material). Of these, 554 were up-regulated and 387 down-regulated. Limitation of the data to twofold and fivefold expression differences produced more manageable lists of 243 genes and 16 genes, respectively. Microarray analysis of *P. aeruginosa* strain H103 cells treated with 1× MIC of ciprofloxacin identified a total of 1,230 genes that were significantly up- and down-regulated. A considerable number of ORFs on both of these lists fell into either the "hypothetical gene" or "putative enzyme" functional categories (27) (52 and 579 ORFs for 0.3× and 1× MIC treatments, respectively), illustrating that a large proportion of genes responsive to ciprofloxacin remain to be fully characterized. Moreover, the expression changes for
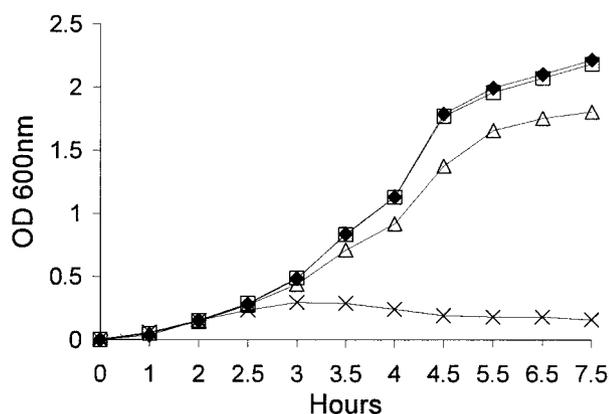
many of these ORFs tended to be fairly large in comparison to the expression changes observed for the annotated genes, highlighting the potential importance of these uncharacterized ORFs in the cellular response of *P. aeruginosa* to ciprofloxacin.

Most striking among the responsive genes was the region between PA0613 and PA0648, where virtually every ORF was increased and where the expression level for ORFs in this region became more pronounced with the increasing concentration of ciprofloxacin (Table 4). PA0616 to PA0648 codes for the R2 and F2 pyocins, which share homology with various bacteriophage-related proteins (21). Both pyocins are known to resemble phage tails and appear to have evolved as bacteriocins rather than being simple defective phages (21). Consistent with this and the observed expression trends, phage tail particles were noted by electron microscopy in the supernatants of wild-type *P. aeruginosa* cells treated with 1× MIC of ciprofloxacin but not in the supernatants of untreated wild-type or ciprofloxacin-treated strain PA0620::*luxCDABE* containing a polar mutation in this R2 pyocin ORF (Fig. 2). The filamentous particles observed in Fig. 2A and C likely represent cleaved flagella, as observed by Higerd et al. (10).

Regulation of this region, as well as that of other *P. aeruginosa* pyocins (i.e., PA0985, PA1150, and PA3866), has been well documented (19) and involves RecA (PA3617) control of the repressor protein PrtR (PA0611), allowing transcription to proceed through interaction of the activator PrtN (PA0610) with the P-box motif (25). Both the regulatory network ORFs and the other pyocins in *P. aeruginosa* were also up-regulated by subinhibitory and inhibitory ciprofloxacin (Table 4), reinforcing the idea that ciprofloxacin induces the expression of pyocins. These observations were consistent with the responsiveness of pyocins and lysogenic phage to mutagenic and/or DNA-damaging events (20), given that ciprofloxacin is known to interfere with DNA replication and thus have mutagenic activity (18, 20).

The array data were validated by analyzing the expression of several R2/F2 pyocin ORFs by relative real-time PCR. Expression trends were found to be similar to those observed for the microarray data (Table 5). Expression changes were also confirmed by measuring over a 3-hour time course in the presence of 0.1×, 0.3×, and 1× MIC of ciprofloxacin the luminescence from mini-Tn5*luxCDABE* transcriptional fusions inserted in the PA0620 (Fig. 3) and PA0641 (data not shown) genes. Similar up-regulation trends to the array data were noted for the DNA gyrase inhibitors ciprofloxacin (Fig. 3) and novobiocin but not for the β-lactam ceftazidime, highlighting the responsiveness of the R2/F2 pyocin region to DNA gyrase inhibition.

Mutants in pyocin/phage region have higher fluoroquinolone MICs. To determine whether these striking expression changes in the pyocin/phage region in response to ciprofloxacin were relevant to susceptibility/resistance, mutants deficient in a number of these genes (Table 1) were tested for antibiotic sensitivity. MICs of all four fluoroquinolones examined were generally ≥8-fold higher for PA0613::IS*lacZ*, PA0620::*lux*, PA0621::IS*lacZ*, and PA0641::*lux* mutants compared to the wild-type *P. aeruginosa* strain H103 (Table 3). No significant changes in the MICs were observed for the control antibiotics amikacin, cefepime, or gentamicin. A *recA*::IS*lacZ* mutant also exhibited eightfold-higher MICs for all four fluoroquinolones,

FIG. 1. Growth curve for *P. aeruginosa* strain H103 in the presence or absence of ciprofloxacin. ◆, untreated PAO1-H103; □, PAO1-H103 plus 0.01 μg/ml ciprofloxacin; △, PAO1-H103 plus 0.03 μg/ml ciprofloxacin; and ×, PAO1-H103 plus 0.1 μg/ml ciprofloxacin.

TABLE 4. Pyocin/phage operon and related genes induced by 0.3× and 1× MIC of ciprofloxacin[a]

ORF	Gene name[b]	Change (n-fold) with 0.3× MIC	Change (n-fold) with 1× MIC	Description
PA0610	prtN	2.0	6.8	Transcriptional activator
PA0611	prtR	1.3	3.0	Transcriptional repressor
PA0612		—[c]	8.5	Homolog of Zn^{2+} finger protein
PA0613		4.0	19.3	Conserved hypothetical
PA0614	Hol	3.3	15.3	Holin
PA0615		1.7	7.2	Conserved hypothetical
PA0616	V_{R2}	2.9	13.0	Homologous to baseplate assembly protein V
PA0617	W_{R2}	3.0	13.9	Homologous to baseplate assembly protein W
PA0618	J_{R2}	2.1	10.0	Homologous to baseplate assembly protein J
PA0619	I_{R2}	3.2	14.7	Homologous to tail protein I
PA0620	H_{R2}	2.7	12.1	Homologous to tail fiber protein H
PA0621		4.3	16.7	Homologous to tail fiber assembly protein
PA0622	FI_{R2}	2.4	10.3	Homologous to contractile sheath protein FI
PA0623	FII_{R2}	2.9	12.8	Homologous to tail tube protein FII
PA0624		2.7	12.8	Conserved hypothetical
PA0625		2.5	14.1	Homologous to tail length determination protein
PA0626	U_{R2}	1.8	9.8	Homologous to tail formation protein U
PA0627	X_{R2}	2.3	10.8	Homologous to tail protein X
PA0628	D_{R2}	1.8	10.0	Homologous to tail formation protein D
PA0629	Lys	2.2	8.7	Lytic protein; homology to predicted chitinase
PA0630		1.6	10.6	Hypothetical protein
PA0631		2.4	16.2	Unique hypothetical protein
PA0632		3.4	21.2	Unique hypothetical protein
PA0633	V_{F2}	4.3	16.4	Homologous to major tail protein V
PA0634		3.2	15.8	Unique hypothetical protein
PA0635		2.0	15.9	Conserved hypothetical protein
PA0636	H_{F2}	2.1	10.0	Homologous to tail length determination protein H
PA0637	M_{F2}	2.7	11.0	Homologous to tail fiber protein M
PA0638	L_{F2}	—	8.5	Homologous to tail fiber protein L
PA0639	K_{F2}	3.0	15.9	Homologous to tail assembly protein K
PA0640	I_{F2}	3.1	16.4	Homologous to tail assembly protein I
PA0641	J_{F2}	2.4	9.8	Homologous to tail protein J
PA0642		2.7	12.9	Hypothetical protein
PA0643		1.9	8.9	Homologous to tail fiber domain protein
PA0644		4.0	18.0	Hypothetical protein
PA0645		1.8	15.8	Hypothetical protein
PA0646		3.1	13.4	Homologous to putative tail fiber protein
PA0647		3.8	23.0	Conserved hypothetical protein
PA0648		—	8.2	Conserved hypothetical protein
PA0985	pys5	4.5	18.1	Pyocin S5
PA1150	pys2	—	5.4	Pyocin S2
PA3617	recA	2.8	4.6	Recombinase for DNA recombination and repair
PA3866	pys4	13.9	51.3	Pyocin S4

[a] Only genes identified as being affected by subinhibitory (0.3 µg/ml) or inhibitory (1 µg/ml) ciprofloxacin relative to untreated strain H103 and that were of interest in this study are included. A list of all genes identified as being affected by this concentration of ciprofloxacin and exhibiting a statistically significant change ($P \leq 0.05$) is available in the supplemental material. Genes are identified by ORF designation, gene name or alternative gene name, and homology description based on the *Pseudomonas* genome project (www.pseudomonas.com).
[b] Genes were named as per Table 1 of Nakayama et al. (21) and reflect the homology observed by these authors to phages P2 and λ.
[c] —, no significant change in expression.

while a mutant in *prtR*, the transcriptional repressor, showed no difference in MICs relative to wild-type strain H103 (Table 3). Together, these surprising results indicate that expression of the R2/F2 pyocin region in the wild-type strains determines (i.e., increases) the intrinsic susceptibility of *P. aeruginosa* to ciprofloxacin and other fluoroquinolones at the MIC.

DISCUSSION

To better address the rise in bacterial resistance and develop novel antimicrobials to combat these infections, we must first better understand the response of an organism to antibiotic challenge. While it has long been believed that the target and/or mechanism of action of certain antimicrobials, like cip-

rofloxacin, is well understood, recent microarray work on transcriptional responses to drugs with the same proposed mechanism of action stands in stark contradiction to this notion. For example, although novobiocin and ciprofloxacin both target DNA gyrase, distinct expression patterns in response to these two agents have been observed in *H. influenzae* and *Bacillus subtilis* (7, 14). Furthermore, the expression signatures of other DNA-damaging compounds, namely, hydrogen peroxide and doxorubicin, but not ethidium bromide, have been shown to be closely related to the expression profiles of quinolone topoisomerase inhibitors (14). Examination of the transcriptional responses of *P. aeruginosa* to subinhibitory and inhibitory ciprofloxacin likewise showed an unanticipated finding, in that increased expression of the R2/F2 pyocin region in response to
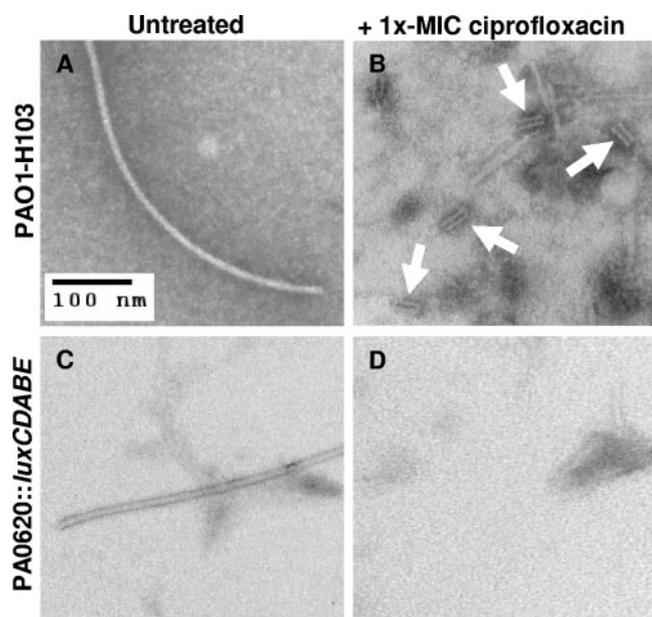
FIG. 2. Electron micrographs of supernatants from *P. aeruginosa* cells that were untreated or treated with 1× MIC of ciprofloxacin. (A) Untreated strain H103 cells. (B) 1× MIC of ciprofloxacin-treated strain H103 cells. Arrows point to the presence of pyocin/phage tail structures. (C) Untreated strain PA0620::*luxCDABE* cells. (D) 1× MIC of ciprofloxacin-treated PA0620::*luxCDABE* cells showing absence of R-type pyocin tail structures.
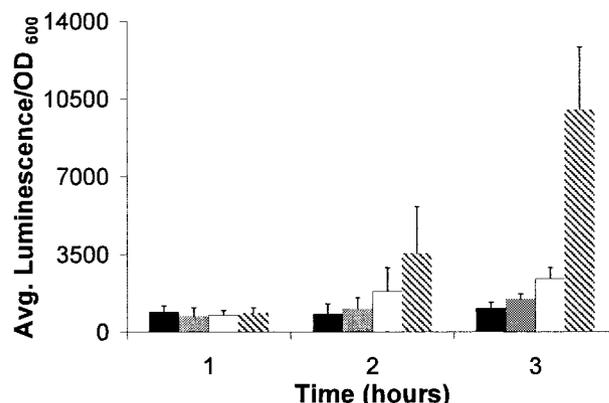

FIG. 3. Induction of PA0620::*luxCDABE* fusions in LB media alone (black bars), with 0.1× MIC of ciprofloxacin (0.01 µg/ml; grey bars), with 0.3× MIC of ciprofloxacin (0.03 µg/ml; white bars), or with 1× MIC of ciprofloxacin (0.1 µg/ml; patterned bars).

treatment with ciprofloxacin at or below the MIC seems to play a critical role in mediating susceptibility to ciprofloxacin and other fluoroquinolones. Thus, in addition to its ability to interact with DNA topoisomerases, ciprofloxacin also induces the expression of this susceptibility determinant. No significant changes in the genes encoding the target DNA gyrase were observed after treatment with 1× MIC of ciprofloxacin, although such changes were not anticipated, since Gmuender et al. (7) observed changes in DNA gyrase target genes only with 10× MIC of ciprofloxacin.

The work of Nakayama et al. (21) provides a possible explanation for how the R2/F2 pyocins may encode a fluoroquinolone susceptibility determinant. In their analysis of the R2/F2 pyocin region, they presented evidence of the existence of a phage lytic system. PA0614 exhibited a hydrophobicity profile

similar to holins of phage P2, φCTX, and λ phage. Holins mediate the translocation of lytic enzymes to the outer membrane. Analysis of PA0629 found weak but significant similarity to chitinases, enzymes which have substrate specificities similar to those of lysozymes which degrade bacterial peptidoglycan, giving rise to the suggestion that PA0629 may be a lytic enzyme required for pyocin secretion (21). Consistent with this proposal, expression of PA0614 and/or PA0629 from an inducible plasmid was shown to result in cell lysis in *Escherichia coli* and *P. aeruginosa* (21). Given this proposed phage lytic system in the R2/F2 pyocin region, it seems plausible that the lethality associated with ciprofloxacin induction of the pyocin region may be due to induction of this lysis system. Indeed, growth of *P. aeruginosa* PAO1-H103 was inhibited by 1× MIC of ciprofloxacin (Fig. 1), and Gram stains of these cultures showed diminished numbers of cells over exposure time (data not shown). Correspondingly, individual mutants in both the R2 and F2 regions (i.e., mutants in genes PA0613, PA0620, PA0621, and PA0641) exhibited resistance to ciprofloxacin (Table 3). These results highlight the importance of cell lysis in R2/F2 pyocin-mediated susceptibility to ciprofloxacin. Conversely, resistance to ciprofloxacin was not observed for mutants in another pyocin gene, PA3866 (Table 3), even though it was even more strongly induced by subinhibitory and inhibitory concentrations of ciprofloxacin, indicating that this phenomenon is specific to the R2/F2 pyocins.

Genomic analysis of many clinical and environmental isolates of *P. aeruginosa* has demonstrated significant mosaicism in the R2/F2 pyocin region (4, 28). Interestingly, all strains studied contained either the R2 pyocin or the F2 pyocin regions, as assessed by microarray analysis (4, 28); the putative holin gene PA0614 and lysis gene PA0629, however, were found to be conserved across all eight clinical isolates studied. Recent work on colicins (*E. coli* equivalent of pyocins) and their role in biodiversity has identified them as having the ability to promote microbial diversity in static or localized environments, in a manner similar to the game rock-paper-scissors (16, 17). In this model, strains that produce colicin kill sensitive strains, which outcompete resistant strains, which, in turn, outcompete colicin-producing strains on the basis of

TABLE 5. Comparison of expression changes for various pyocin/phage-related open reading frames as analyzed by relative real-time PCR and custom *P. aeruginosa* microarray

ORF	Gene Name	Change (*n*-fold) in PAO1 strain H103 at 0.3× MIC of ciprofloxacin	
		Custom microarray	Real-time PCR
PA0610	*prtN*	2.0	10.0
PA0611	*prtR*	1.3	11.4
PA0621		4.3	3.0
PA0623	*FII$_{R2}$*	2.9	3.7
PA3617	*recA*	2.8	3.9
PA3866	*pys3*	13.9	22.5

growth rate advantages. However, in well-mixed or large spatial environments, the resistant strain will predominate over time (16). While this work does not demonstrate genomic loss of the pyocin region, it does indicate that there is a selective pressure favoring colicin-resistant strains. Similarly, progressive loss of pyocin production in cystic fibrosis clinical isolates has been observed (11, 24) and occurs while patients are under selective pressure, as antimicrobial therapy is applied in an attempt to control their lung disease. It is intriguing to speculate that parts or all of the pyocin region can be deleted in *P. aeruginosa* clinical isolates due to strain competition caused by enhanced survival of the pyocin-resistant strain and that this deletion is further advantageous with respect to the fluoroquinolone resistance profile of the organism. Further studies to investigate this prospect are required, since their findings have important implications in the role subinhibitory antimicrobials play in the development of resistance in *P. aeruginosa*.

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