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Complex Ciprofloxacin Resistome Revealed by Screening a *Pseudomonas aeruginosa* Mutant Library for Altered Susceptibility[▽]

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Pseudomonas aeruginosa **offers substantial therapeutic challenges due to its high intrinsic resistance to many antibiotics and its propensity to develop mutational and/or adaptive resistance. The PA14 comprehensive mutant library was screened for mutants exhibiting either two- to eightfold increased susceptibilities (revealing genes involved in intrinsic resistance) or decreased susceptibilities (mutational resistance) to the fluoroquinolone ciprofloxacin. Thirty-five and 79 mutants with increased and decreased susceptibilities, respectively, were identified, as confirmed by broth dilution.**

Pseudomonas aeruginosa is a major cause of hospital infections and is the pathogen most commonly associated with mortality in cystic fibrosis patients. Infections with this organism are very difficult to treat due to its high intrinsic antibiotic resistance. Fluoroquinolones, such as ciprofloxacin, are broad-spectrum antimicrobials that target the bacterial enzymes DNA gyrase and topoisomerase IV (6) and are clinically used to treat chronic *P. aeruginosa* infections in cystic fibrosis patients (5, 9). The massive use of fluoroquinolones has led to increased quinolone resistance in *P. aeruginosa* (18) as well as multidrug resistance, a serious problem in the clinic. Certain mutations that lead to ciprofloxacin supersusceptibility (e.g., *lon* [1]) or resistance (e.g., mutations in the genes for the target topoisomerase enzymes or the upregulation of efflux pump expression [10]) are already known. In addition, subinhibitory concentrations of fluoroquinolones play an important role in the development of resistance, in that *P. aeruginosa* cultures pretreated with subinhibitory concentrations of ciprofloxacin develop an adaptive resistance phenotype (1).

It has been proposed that antibiotics rarely have a simple mechanism of action. Microarray studies indicate that they lead to the upregulation of dozens to hundreds of genes when they are used at concentrations at or near the MIC (3, 4, 14). It has been suggested that some of these genes are involved in target inhibition, others are involved with the induction of cellular stress pathways, and some are involved with defensive measures that the bacterium takes to resist the actions of antibiotics. In this view of antibiotic action, it seems likely that there should be many more genes that are involved in increased or decreased susceptibility in bacteria than have previously been supposed and that some of these might have clinical relevance.

Therefore, in the present study we screened a comprehensive PA14 transposon mutant library (13) for mutants that showed either increased susceptibility (reflecting intrinsic resistance) or decreased susceptibility (reflecting mutational resistance) to ciprofloxacin as a survey to reveal the extent of the ciprofloxacin resistome. Freshly diluted overnight cultures were used to inoculate approximately 3×10^4 cells per spot on to Mueller-Hinton agar plates containing ciprofloxacin at 0.025 μg/ml (half the MIC for the parent strain) to screen for increased susceptibility and at 0.2 μg/ml to screen for decreased susceptibility. The mutants identified by this approach were confirmed to show a changed susceptibility by determination of the MICs (17), and changes as small as twofold were taken into account. We acknowledge that twofold changes in MICs are generally considered within the error of the standard assessment protocols. However, only results for those mutants for which we could consistently confirm changes in at least three independent measurements are shown. Growth was measured in liquid medium at 37°C by using a Tecan Spectrafluor Plus apparatus by measuring the absorbance at 620 nm every 18 min for 24 h under shaking conditions.

The first ciprofloxacin screen at 0.025 μg/ml yielded a total of 62 mutants with an inability to grow in the plate assay, leading to 28 confirmed mutants with an increase in susceptibility (Table 1). An additional seven mutants were identified by specifically measuring the MIC of ciprofloxacin for mutants with mutations in the same operon as that for the mutants that appeared in the screen, for a total of 35 mutants (Fig. 1; Table 1). Mutants with mutations located in an operon might exhibit polar effects.

The majority of these mutants demonstrated only twofold changes in susceptibility, although a mutant with a mutation in *ftsK* was eightfold more susceptible. Noticeable among the ciprofloxacin mutants with increased susceptibility was the number of mutants with mutations that were involved in DNA replication and repair, such as the Holliday junction helicase *ruvA*, the ATP-dependent RNA helicase *recG*, the recombinase *xerD*, and the site-specific recombinase *sss*. In addition, we observed the major intrinsic multidrug efflux pump *mexAB*-

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TABLE 1. Changes in susceptibility of confirmed *P. aeruginosa* mutants showing increased susceptibility

PA no.[a]	Gene name	Gene description	Gene class(es)[b]	Fold increased susceptibility	Growth rate[c]	Regulation by ciprofloxacin[d]
PA0334		Putative MFS transporter	MP, TSM	2	++	
PA0336	*ygdP*	Dinucleoside polyphosphate hydrolase	NB	2	++	↑*#
PA0337	*ptsP*	Phosphoenolpyruvate-protein phosphotransferase	TSM	2	++	
PA0338		Hypothetical protein	HYP, M/A	2	++	↑*#
PA0425	*mexA*	RND multidrug efflux membrane fusion protein	TSM, AB	2	++	
PA0426	*mexB*	RND multidrug efflux transporter	TSM, AB	2	++	↑*
PA0427	*oprM*	Major intrinsic multiple-antibiotic-resistance efflux outer membrane protein OprM	AB, MP, TSM	2	++	↑#
PA0702		Hypothetical protein	MP, HYP	2	++	
PA0703		Probable major facilitator superfamily transporter	MP, TSM	2	++	
PA0966	*ruvA*	Holliday junction DNA helicase	DRR	2	(++)	
PA1098	*fleS*	Two-component sensor	TCRS	2	(++)	
PA1375	*pdxB*	Erythronate-4-phosphate dehydrogenase	CCC, others	2	+	
PA1588	*sucC*	Succinyl coenzyme A synthetase beta subunit	EM	2	+	
PA1611		Putative sensor/response regulator hybrid	TR, TCRS	4	+	↑*
PA1667		Hypothetical protein	HYP	2	+	
PA1777	*oprF*	Major porin and structural outer membrane porin	MP, TSM	2	+	↓#
PA1800	*tig*	Trigger factor	CD, HSP	2	++	↓#
PA1801	*clpP*	ATP-dependent Clp protease proteolytic subunit	HSP	2	+	
PA1802	*clpX*	ATP-dependent Clp protease ATP-binding subunit	HSP	2[e]	++	
PA1803	*lon*	Lon protease	A/P, TM	4	++	
PA2432		Putative transcriptional regulator	TR, others	2	++	
PA2549		Hypothetical protein	MP, HYP	2	+	
PA2615	*ftsK*	Cell division/stress response protein	CD	8	+	
PA3516		Probable lyase	PE	2	++	
PA3517		Putative lyase	CCC	2	++	
PA3738	*xerD*	Integrase/recombinase *xerD*	DRR	4	+	
PA4459		Hypothetical protein	HYP	2	++	↑#
PA4667		Hypothetical protein	HYP	2	++	
PA4685		Hypothetical protein	HYP	2	+	↑*
PA4781		Putative two-component response regulator	TR, TCRS	2	+	
PA5253	*algP*	Alginate regulatory protein AlgP	TR	2	++	
PA5280	*sss*	Site-specific recombinase	DRR	4	+	
PA5345	*recG*	ATP-dependent DNA helicase RecG	DRR	4	++	
PA5366	*pstB*	Phosphate ABC transporter, ATP-binding protein	MP, TSM	2	+	
PA5375	*betT1*	Choline/carnitine/betaine transporter family protein	MP, TSM	2	++	↓*#

[a] Note that these are the original assignments for these gene mutations and were not confirmed again in this study, although the mutants were used as obtained with minimal subculture. We could also confirm 10 mutations in operons or an overlap between libraries. The mutants with the underlined mutations were tested after the initial screen due to the presence of mutations in an operonic relationship with genes that, when they were altered, influenced susceptibility to ciprofloxacin.

[b] A/P, adaptation, protection; TSM, transport of small molecules; TM, translation, posttranslational modification; PE, putative enzyme; TR, transcriptional regulator; HYP, hypothetical protein; MP, membrane protein; EM, energy metabolism; DRR, DNA replication recombination; CCC, carbon compound metabolism; CD, cell division; NB, nucleotide biosynthesis and metabolism; M/A, motility and attachment; AB, antibiotic resistance and susceptibility; TCRS, two-component regulatory systems; HSP, chaperones and heat shock proteins.

[c] ++, normal growth rate compared to that of the wild type; (++), normal growth rate compared to that of the wild type but reduced yield; +, reduced growth rate compared to that of the wild type.

[d] Data are from previous reports (1, 2). All *P. aeruginosa* genes were differentially expressed by treatment with 0.3× MIC (*) and 1× MIC (#) of ciprofloxacin. The direction of the arrow indicates the trend of expression relative to that by untreated cells.

[e] Change in susceptibility observed after 48 h.

oprM, the recently identified *lon* mutant (1), and three other genes (namely, *tig*, *clpP*, and *clpX*) in the same operon. *clpP* and *clpX* encode ATP-dependent proteases like Lon. Not surprisingly, a few mutants (e.g., *sss*, *xerD*, *ftsK*, and *ruvA* mutants) showed somewhat slower growth than the wild-type strain PA14. However, all of these mutants remained more susceptible even after 48 h, while several of the other slower-growing mutants tested were not more susceptible to ciprofloxacin, indicating that slow growth did not cause increased ciprofloxacin susceptibility per se.

The constellation of genes involved in intrinsic resistance is demonstrated in Fig. 1, which indicates that these genes were spread throughout the chromosome. Although the high-throughput nature of this screen did not permit us to complement each mutant, we were able to confirm the increased susceptible phenotype with several independent isolates from

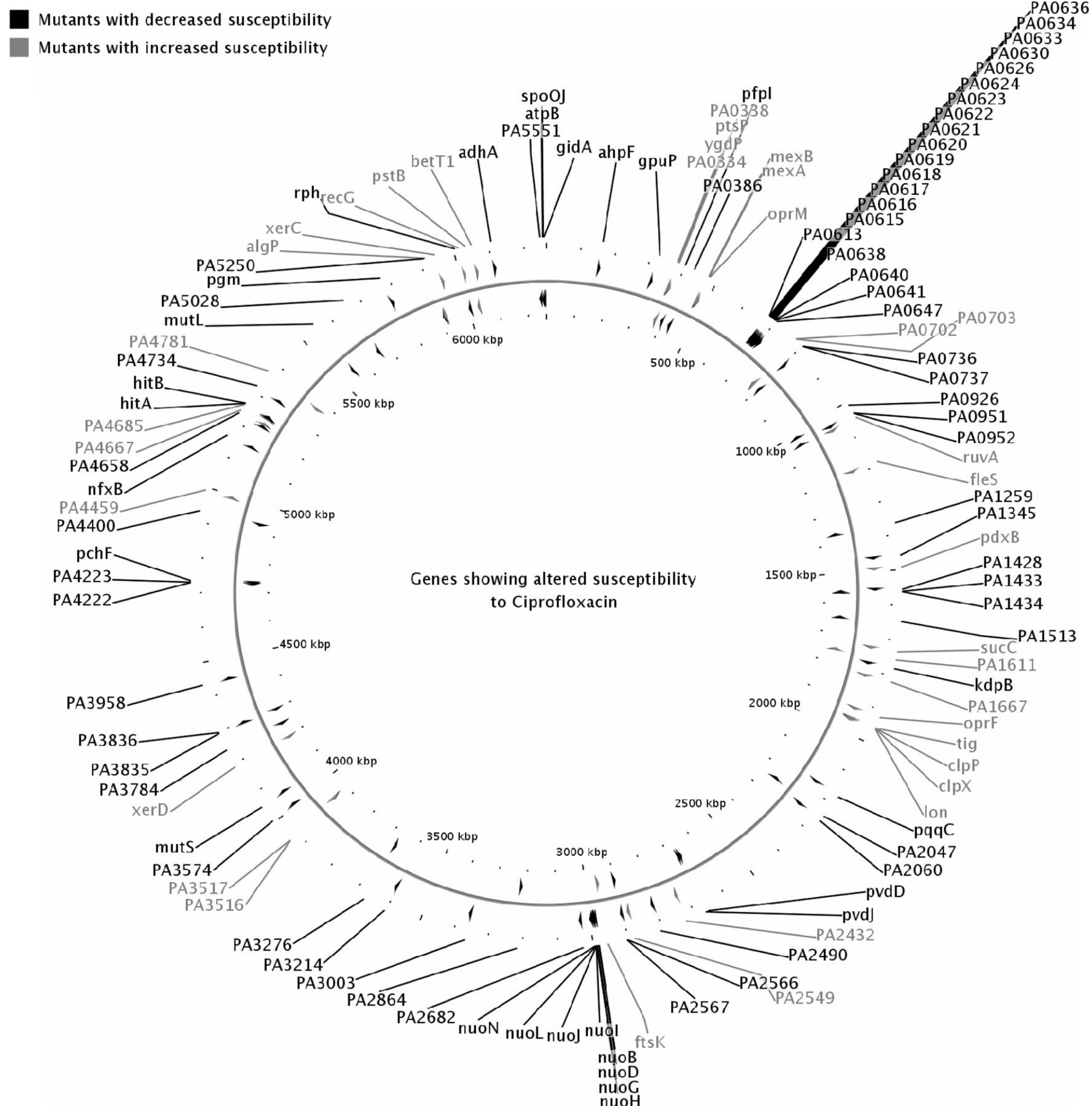

FIG. 1. Distribution of mutations around the genome. The genome image was generated by using CGView (16).

the PAO1 *lux* mutant library (12) and also confirmed the existence of multiple mutants with mutations in five operons. Interestingly, as predicted, we were able to demonstrate that 10 of the genes giving rise to an increase in susceptibility upon transposon mutation were among those differentially expressed in response to ciprofloxacin on our previous microarrays (2). Even though the mutant with a mutation in *clpP* showed only a twofold altered susceptibility, it could be successfully complemented with the cloned gene. We also successfully complemented with the cloned gene the mutant that had a *fleS* mutation that showed a stable twofold increase in susceptibility to ciprofloxacin and norfloxacin compared to that of the wild-type strain PAO1 and for which the killing rate by ciprofloxacin was increased compared to that for the wild-type strain PAO1. FleS is known to be involved in the regulation of flagellum biosynthesis (15); and we were also able to complement a major defect in swarming motility, an ability to swarm somewhat on swimming medium, and an observed 60% decrease in static biofilm formation compared to that by wild-type strain PAO1 (data not shown).

Screening of the library for mutants that showed at least a twofold decrease in susceptibility to ciprofloxacin identified 46 mutants. A further 13 mutants with mutations in adjacent genes in operons and 20 mutants with phage-related mutations were included, for a total of 79 mutants with decreased susceptibility (Fig. 1; Table 2). It is worth noting that such a high-throughput approach is applicable only for genes for which the complete loss of the protein is practical (i.e., nonessential genes). Indeed, nearly all mutants with decreased susceptibility tested were able to grow as well as the wild type (Table 2). We were able to identify previously known genes such as the *mexCD-oprJ* efflux regulator *nfxB*, mutators *mutS* and *mutL*, and the phage-related mutations and observed multiple mutations in nine operons, including the *nuoD* NADH

TABLE 2. Changes in susceptibility of confirmed *P. aeruginosa* mutants showing decreased susceptibility

PA no.[a]	Gene name	Gene description	Gene class(es)[b]	Fold decreased susceptibility	Growth rate[c]	Regulated by ciprofloxacin[d]
PA0140	*ahpF*	Alkyl hydroperoxide reductase subunit F	A/P	4	++	
PA0287		Putative sodium:solute symporter	TSM	2	++	
PA0355	*pfpI*	Protease	TM	2[e]	++	
PA0386		Putative oxygen-independent coproporphyrinogen III oxidase	PE	2	++	
PA0613[e]		Conserved hypothetical protein	HYP, PR	2	++	↑ *#
PA0615		Conserved hypothetical protein	HYP, PR	2	++	↑ *#
PA0616		Hypothetical protein	HYP PR	2	++	↑ *#
PA0617		Putative base plate assembly protein W	PR	2[e]	++	↑ *#
PA0618		Putative phage base plate assembly protein	PR	2	++	↑ *#
PA0619		Putative phage tail protein	PR	2	++	↑ *#
PA0620		Putative tail fiber protein	PR	2	++	↑ *#
PA0621		Putative tail fiber assembly protein	PR	2	++	↑ *#
PA0622		Putative phage tail sheath protein	PR	1.5[e]	++	↑ *#
PA0623		Putative phage tail tube protein	PR	2	++	↑ *#
PA0624		Conserved hypothetical protein	PR	2	++	↑ *#
PA0626		Putative tail formation protein	PR	2	++	↑ *#
PA0630		Hypothetical protein	PR	2	++	↑ *#
PA0633		Putative major tail protein V	PR	2	++	↑ *#
PA0634		Hypothetical protein	PR	2	++	↑ *#
PA0636		Putative tail length determination protein	PR	2	++	↑ *#
PA0638		Putative minor tail protein L	PR	2	++	↑ #
PA0640		Putative phage tail assembly protein	PR	1.5	++	↑ *#
PA0641		Putative phage-related protein, tail component	PR	2–4	++	↑ *#
PA0647		Conserved hypothetical protein	PR	2	++	↑ *#
PA0736-PA0737		Hypothetical protein	HYP	2	++	↑ *#
PA0926		Hypothetical protein	HYP	4	++	
PA0951-PA0952		Hypothetical protein	HYP	4	++	
PA1259		Conserved hypothetical protein	HYP	2[e]	++	
PA1345	*gshB*	Glutathione synthase	HYP	2	++	
PA1428		Hypothetical protein	HYP	2	++	
PA1433		Conserved hypothetical protein	MP	2[e]	++	↑ #
PA1434		Putative periplasmic protein	HYP	2	++	
PA1513		Hypothetical protein	MP, HYP	2	++	
PA1634	*kdpB*	Potassium-transporting ATPase, B chain	TSM	2[e]	++	
PA1987	*pqqC*	Pyrroloquinoline biosynthesis protein C	BC	2	++	
PA2047		AraC family transcriptional regulator	TR	8	++	
PA2060		Putative permease of ABC transporter	TSM	2[e]	++	↑ *
PA2399	*pvdD*	Pyoverdine synthetase D	SF A/P	2	++	
PA2400	*pvdJ*	Pyoverdine synthesis protein	A/P	2[e]	++	
PA2490		Conserved hypothetical protein	HYP	4	++	
PA2566-PA2567		Hypothetical protein	HYP	2	++	
PA2638	*nuoB*	NADH dehydrogenase I chain B	EM	2[e]	++	
PA2639	*nuoD*	NADH dehydrogenase I chains C and D	EM	2[e]	++	
PA2642	*nuoG*	NADH dehydrogenase I chain G	EM	2[e]	++	↓ #
PA2643	*nuoH*	NADH dehydrogenase I chain H	EM	2	++	
PA2644	*nuoI*	NADH dehydrogenase I chain I	EM	2	++	
PA2645	*nuoJ*	NADH dehydrogenase I chain H	EM	2	++	
PA2647	*nuoL*	NADH dehydrogenase I chain L	EM	2[e]	++	
PA2649	*nuoN*	NADH dehydrogenase I chain N	EM	2[e]	++	↑ *
PA2682		Putative dienelactone hydrolase	PE	2[e]	++	
PA2864		Hypothetical protein	HYP	2	++	
PA3003		Conserved hypothetical protein	HYP	2	++	
PA3214		Hypothetical protein	HYP	2	++	
PA3276		Hypothetical protein	HYP	2[e]	++	↓ *
PA3574		TetR family transcriptional regulator	TR	2	++	
PA3620	*mutS*	DNA mismatch repair protein MutS	DRR	4	++	
PA3784		Conserved hypothetical protein	HYP	2[e]	++	↑ #
PA3835-PA3836		Hypothetical protein	HYP	2	++	
PA3958		Possible nuclease or phosphatase	HYP	2[e]	++	↑ *#
PA4222		Putative ATP-binding component of ABC transporter	TSM	4	(++)	
PA4223		Putative ATP-binding component of ABC transporter	MP, TSM	4	(++)	

Continued on following page

TABLE 2—*Continued*

PA no.[a]	Gene name	Gene description	Gene class(es)[b]	Fold decreased susceptibility	Growth rate[c]	Regulated by ciprofloxacin[d]
PA4225	*pchF*	Pyochelin synthetase	SF, TSM	2	++	
PA4400		Probable pyrophosphohydrolase	DRR	4	++	
PA4600	*nfxB*	Transcriptional regulatory protein	TR	4	++	
PA4658		Conserved hypothetical protein	HYP	2[e]	++	
PA4687	*hitA*	Ferric iron-binding periplasmic protein	TSM	2	++	
PA4688	*hitB*	Iron (III) transport system permease HitB	MP, TSM	2[e]	++	↑ *#
PA4734		Conserved hypothetical protein	HYP	2	++	
PA4946	*mutL*	DNA mismatch repair protein MutL	DRR	4	++	↑ #
PA5028		Hypothetical protein	HYP	4	++	
PA5131	*pgm*	Phosphoglycerate mutase	CCC	2	++	
PA5250		Putative integral membrane transport protein	MP, HYP	2[e]	++	
PA5334	*rph*	RNase PH	T	2[e]	++	
PA5427	*adhA*	Alcohol dehydrogenase, zinc containing	EM, CCC	4	++	↓ #
PA5551		Hypothetical	HYP	2	++	
PA5560	*atpB*	ATP synthase A chain	EM	2	++	↑ #
PA5562	*spoOJ*	Chromosome partitioning protein Spo0J	CD	2[e]	++	
PA5565	*gidA*	Glucose-inhibited division protein A	CD	2	+	↑ #
PA14_ 46620		Pyridine nucleotide-disulfide oxidoreductase	PE	2–4	++	

[a] Note that these are the original assignments for these gene mutations and were not confirmed again in this study, although the mutants were used as obtained with minimal subculture. We could also confirm 39 mutations in operons or an overlap between libraries. The mutants with the underlined mutations were tested after the initial screen due to the presence of mutations in an operonic relationship with genes that, when they were altered, influenced susceptibility to ciprofloxacin.

[b] A/P, adaptation, protection; TSM, transport of small molecules; TM, translation; posttranslational modification; PE, putative enzyme; TR, transcriptional regulator; HYP, hypothetical protein; PR, related to phage; MP, membrane protein; BC, biosynthesis of cofactors; SF, secreted factors; EM, energy metabolism; DRR, DNA replication recombination; CCC, carbon compound metabolism; T, transcription; CD, cell division.

[c] ++, normal growth rate compared to the wild type; (++), normal growth rate compared to that of the wild type but reduced yield; +, reduced growth rate compared to that of the wild type.

[d] Data are from previous reports (1, 2). All *P. aeruginosa* genes were differentially expressed by treatment with 0.3× MIC (*) and 1× MIC (#) of ciprofloxacin. The direction of the arrow indicates the trend of expression relative to that for untreated cells.

[e] Change in susceptibility observed after 48 h.

dehydrogenase operon. We also observed mutations in several iron transport genes, consistent with recent views suggesting roles for free radicals in antibiotic killing (7, 11). Among the genes giving rise to decreased susceptibility upon transposon mutation, we found 32 that were differentially expressed in response to ciprofloxacin (2). It should be noted that while the library was quite comprehensive, it was not complete; e.g., a mutation in the *mexS* gene that regulates the MexEF-OprN efflux operon was not available.

The resistome comprises all genes that, when they are mutated, give rise to altered susceptibility. The present survey indicates for the first time that the resistome for ciprofloxacin in *P. aeruginosa* is very large; i.e., it comprises more than 100 genes. It is important to note that while we have not demonstrated that these mechanisms are clinically relevant, they do indicate the enormous gene pool that can influence susceptibility to this antibiotic class. Where this may become important is in understanding two complex clinical phenomena, namely, MIC creep (in which the background level of intrinsic resistance to a given antibiotic in a population of clinical isolates rises over time) and adaptive resistance (in which the level of resistance is affected by environmental factors, such as growth in vivo or exposure to antibiotics at sub-MIC). MIC creep has previously been supposed to represent the accumulation of mutations over time (5, 8) and differs from obvious clinical resistance, which is caused by breakthrough mutations (e.g., efflux pump overexpression mutations) that cause very large changes in susceptibility. Adaptive resistance has also been proposed to represent a complex phenomenon in which multiple genes that influence gene expression can combine to induce resistance. Indeed, no fewer than 43 of the genes that gave rise to altered ciprofloxacin susceptibility were included in the list of those that are differentially expressed in *P. aeruginosa* in the presence of ciprofloxacin (2), which is known to promote adaptive resistance to itself. By performing a broad survey, as described here, we have provided much food for thought, and it will be essential in future studies to follow up these observations with detailed studies to determine if these candidate mutations are indeed relevant to clinically meaningful antibiotic resistance.

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