

Mutations That Enhance the Ciprofloxacin Resistance of *Escherichia coli* with *qnrA1*

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Plasmid-mediated *qnr* genes provide only a modest decrease in quinolone susceptibility but facilitate the selection of higher-level resistance. In *Escherichia coli* strain J53 without *qnr*, ciprofloxacin resistance often involves mutations in the GyrA subunit of DNA gyrase. Mutations in *gyrA* were absent, however, when 43 mutants with decreased ciprofloxacin susceptibility were selected from J53(pMG252) with *qnrA1*. Instead, in 13 mutants, individual and whole-genome sequencing identified mutations in *marR* and *soxR* associated with increased expression of *marA* and *soxS* and, through them, increased expression of the AcrAB pump, which effluxes quinolones. Nine mutants had increased expression of the MdtE efflux pump, and six demonstrated increased expression of the *ydhE* pump gene. Many efflux mutants also had increased resistance to novobiocin, another pump substrate, but other mutants were novobiocin hypersusceptible. Mutations in *rfaD* and *rfaE* in the pathway for inner core lipopolysaccharide (LPS) biosynthesis were identified in five such strains. Many of the pump and LPS mutants had decreased expression of OmpF, the major porin channel for ciprofloxacin entry. Three mutants had increased expression of *qnrA* that persisted when pMG252 from these strains was outcrossed. *gyrA* mutations were also rare when mutants with decreased ciprofloxacin susceptibility were selected from *E. coli* J53 with *aac(6′)-Ib-cr* or *qepA*. We suggest that multiple genes conferring low-level resistance contribute to enhanced ciprofloxacin resistance selected from an *E. coli* strain carrying *qnrA1*, *aac(6′)-Ib-cr*, or *qepA* because these determinants decrease the effective ciprofloxacin concentration and allow more common but lower-resistance mutations than those in *gyrA* to predominate.

The plasmid-mediated quinolone resistance (PMQR) gene *qnrA1* causes only a modest decrease in ciprofloxacin susceptibility but facilitates the selection of mutants with higher levels of ciprofloxacin resistance (1). In *Escherichia coli*, ciprofloxacin resistance results from mutations in the quinolone resistance-determining region (QRDR) of the GyrA subunit of DNA gyrase and mutations altering the regulation of efflux pumps, especially AcrAB (2, 3). Gyrase mutations are frequent in ciprofloxacin-resistant mutants of *E. coli* J53 without *qnr* but are surprisingly rare in its presence. Cesaro et al. found that only 2 of 200 mutants selected from J53 with *qnrA* had *gyrA* mutations, while 27% of those selected from *qnr*-free *E. coli* J53 or KL16 had *gyrA* mutations (4). The aim of this study was to explore the reason for the lack of *gyrA* mutations and to characterize the causes of non-*gyrA*-mediated enhanced ciprofloxacin resistance. A variety of mutations affecting susceptibility to ciprofloxacin and other antimicrobials was discovered.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* J53 (*met pro* azide resistant) (5) and plasmids pMG252 IncA-C, conferring resistance to β-lactams (due to *bla*_{FOX-5} and *bla*_{PSE-1}), chloramphenicol (*cmlA1* and *catB2*), gentamicin, kanamycin, quinolones (*qnrA1*), streptomycin (*aadA2*), sulfonamide (*sulI*), tobramycin, trimethoprim (*dhfr*), and mercuric chloride (*mer*) (1); pMG253 (*qnrA1* from pMG252 cloned into pBC SK [*catI*]) (Agilent, Santa Clara, CA) (6); pMG320 [*aac(6′)-Ib-cr* cloned into pBC SK] (7); pAT851 with cloned *qepA* (8); and R692 (Ap Su Tc IncA-C) (9) were used. *E. coli* C600 (B1 *leu thr*) was used as the recipient for outcrossing from J53(pMG252) Cip^r mutants. Spontaneous mutants of J53, J53(pMG252), J53(pMG253), J53(pMG320), and J53(pAT851) were selected with an inoculum of up to ~10¹⁰ organisms on Mueller-Hinton agar plates containing ciprofloxacin at concentrations between the MIC and the mutant prevention concentration (MPC) of each strain after incubation at 37°C for 72 h.

Displacement of pMG252 by incompatibility. *E. coli* C600(R692) was mated with J53(pMG252) Cip^r mutants with selection on media with growth requirements of the recipient but not the donor and tetracycline at 25 μg/ml, to which the donor but not the recipient was resistant. Purified colonies were shown to lack resistances specific for pMG252 and tested for ciprofloxacin MICs.

Susceptibility testing. MICs of ciprofloxacin, nalidixic acid, and novobiocin (Sigma-Aldrich) were determined by agar dilution on Mueller-Hinton agar at 37°C with an inoculum of ~10⁴ CFU according to CLSI guidelines (10). *E. coli* J53 and ATCC 25922 were used for quality control. Minimal bacterial concentrations (MBCs) for ciprofloxacin were determined by subculturing 200 μl from a microdilution well without visible growth after washing with Mueller-Hinton broth onto antibiotic-free Mueller-Hinton agar (10).

PCR and DNA sequencing. We amplified by PCR and sequenced the QRDRs of the *gyrA*, *gyrB*, and *parC* genes (and, in some mutants, the entire genes); regulator genes such as *marR*, *robA*, *acrR*, *soxR*, *soxS*, and *hns* and the promoter of *gadE*; the *lon* protease gene; the efflux pump genes *acrAB*, *acrEF*, *acrD*, *acrS*, and *mdtF* and its promoter; *ydhE*, also with its promoter; the lipopolysaccharide (LPS) biosynthesis genes *rfaE*, *rfaH*, *lpcA*, and *gmhB* and the *rfaDFCL* and *rfaQGPSBIJYZU* operons; and other genes, including *yooA*, *surA*, *topB*, and *rbsA*. DNA templates were prepared by boiling, and the primers used are listed in Table S1 in the sup-

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plemental material. We used Maxima Hot Start PCR master mix (Thermo Scientific, Waltham, MA) in a final volume of 50 μ l. PCR products were purified by using a PCR purification kit (Qiagen, Valencia, CA) and sent for sequencing by the Tufts University Core Facility, Boston, MA.

Whole-genome sequencing. Whole-genome sequencing was performed for 50 mutants by different facilities. Forty mutants (and the parental *E. coli* J53 strain) were sequenced by the Tufts University Genomics Core Facility. Genomic DNA was processed by using the Nextera XT DNA library preparation kit (Illumina). Libraries were size selected with fragment sizes of ≥ 500 bases recovered. The molar concentration of each library was determined with a fragment analyzer (Advanced Analytical), and libraries were mixed into a single pool at equal molar concentrations. The molar concentration of the pooled libraries was adjusted to 8.5 pM for sequencing on an Illumina MiSeq platform using a MiSeq V2 500 cycle kit, yielding 26.34 million pass-filter-paired reads. Five mutants were analyzed by BaseClear BV (Leiden, the Netherlands) on the Illumina HiSeq2500 platform after library preparation with the Nextera XT kit, and five others [including the J53(pMG252) parental strain] were analyzed by AstraZeneca (Waltham, MA), where they also constructed the libraries by using the Nextera XT kit. Libraries were then quantitated against a quantitative PCR (qPCR)-generated standard curve by Kapa BioSystems (Woburn, MA) to perform sequencing on an Illumina MiSeq platform with the V2.2 \times 150 paired-end system. From each source, the raw data in FASTQ format were analyzed with CLC Genomics Workbench software (version 7.5) (CLC Bio, Aarhus, Denmark). After trimming of the sequences, the data were mapped to an *E. coli* J53 reference genome (GenBank accession number [AICK01000001.1](#)) to perform both basic and fixed ploidy variant detection. Variants were filtered for nucleotide changes producing amino acid alterations that occurred in $>80\%$ of the countable fragments. If no mutations were found, the frequency was lowered. All detected mutations were confirmed by conventional PCR and DNA sequencing using primers listed in Table S1 in the supplemental material.

Relative expression levels of genes encoding efflux pumps and regulators. Reverse transcription followed by real-time qPCR (RT-qPCR) was used to determine the expression levels of selected efflux pump genes, including *acrA*, *mdfA*, *yhjE*, *acrE*, *tolC*, *mdtE*, *mdtF*; regulators such as *marA*, *soxS*, *fis*, *dsrA*, and *evgA*; and the porin protein-encoding genes *ompC* and *ompF*. Comparison was made to the expression level of the housekeeping gene *mdh*. Primers used are shown in Table S1 in the supplemental material. The same primers were also employed to generate PCR DNA products to perform qPCR standard plots for each gene tested.

Cultures were inoculated from -80°C stocks into LB broth, grown at 37°C overnight, diluted 1:100 into fresh LB broth, and grown for 2 to 3 h to an optical density at 600 nm (OD_{600}) of ~ 0.3 (Genesys 20 visible spectrophotometer). Total RNA in the cultures was stabilized by using RNeasy Protect Bacterial reagent (Qiagen), bacteria were digested with lysozyme-proteinase K, and total RNA was isolated by using an RNeasy minikit (Qiagen) and On-Column DNase I digestion (Qiagen) according to the manufacturer's specifications. RNA purity and concentration were determined by using a NanoDrop 1000 spectrophotometer (Thermo Scientific). Reverse transcription was performed by using a Verso cDNA synthesis kit (Thermo Scientific). The cDNAs obtained were measured after 40 cycles by using a CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA) with 20- μ l reaction mixtures containing primers (500 nM each), cDNA (3 μ l), and SsoFast EvaGreen supermix from Bio-Rad (10 μ l). To calculate the relative expression levels, the threshold cycle (C_T) value of the target gene was normalized to the C_T value of the housekeeping gene, after the ΔC_T value of the test sample was normalized to the ΔC_T value of the parental strain, and finally, the expression ratio was calculated with the formula $2^{-\Delta\Delta C_T}$. At least three different assays with three independent cultures and RNA extractions were performed for each gene tested.

Assay for promoter efficiency. The wild type and putative promoter mutations were cloned into plasmid pRS415 (11) upstream from its pro-

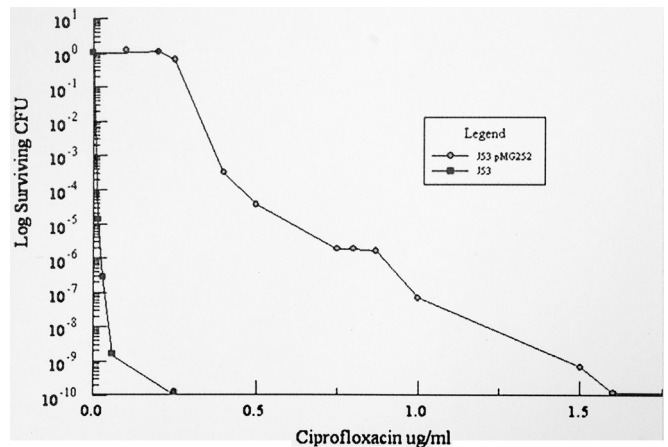


FIG 1 Survival of *E. coli* J53 and J53(pMG252) with increasing ciprofloxacin concentrations. A large inoculum (10^{10} CFU) and appropriate dilutions were applied to Mueller-Hinton agar plates containing the indicated concentrations of ciprofloxacin, and surviving colonies were counted after incubation for 72 h at 37°C . (Reproduced from reference 56.)

moter-free *lac* gene, and comparative β -galactosidase activity in $\Delta lacZ$ *E. coli* strain BW25113 was assayed (12) as a measure of promoter efficiency.

RESULTS

When *E. coli* J53 or J53(pMG252) is plated onto increasing concentrations of ciprofloxacin above its MIC, the number of survivors progressively decreases (Fig. 1). The lowest concentration at which no mutants are observed is the mutant prevention concentration (MPC) (13). It represents the MIC of the least susceptible single-step mutant. For quinolone-susceptible strain J53, the ciprofloxacin MIC was 0.016 $\mu\text{g}/\text{ml}$, and the MPC was 16-fold higher at 0.25 $\mu\text{g}/\text{ml}$. In contrast, J53 containing plasmid pMG252 encoding QnrA1 had a ciprofloxacin MIC of 0.25 to 0.5 $\mu\text{g}/\text{ml}$ and an MPC of between 1 and 2 $\mu\text{g}/\text{ml}$. The shape of the survival curves (Fig. 1) suggests that a variety of mutations provide lower-level resistance and that progressively fewer mutations confer higher-level resistance.

At 3 times the MIC, most (14 of 15 analyzed) of the ciprofloxacin-resistant mutants selected from J53 had alterations in the QRDR of *gyrA*. In contrast, none of the >50 spontaneous ciprofloxacin-resistant mutants selected at 3 times the MIC or other multiples from J53(pMG252) had QRDR mutations in *gyrA* or other topoisomerase genes, nor did any of the 20 ciprofloxacin-resistant mutants selected at up to three times the MIC from J53(pMG252) containing the cloned *qnrA1* gene. This lack of *gyrA* mutations was not *qnr* specific but occurred with other PMQR genes. Mutations in *gyrA* were found in only 2 of 9 mutants selected from J53 with pMG320 containing *aac(6')Ib-cr* at concentrations of ciprofloxacin up to 20 times the MIC and in only 1 of 10 mutants selected at 3 times the ciprofloxacin MIC from J53(pAT851) carrying *qepA*. In every case, mutants from the cultures with the highest concentration of ciprofloxacin with surviving colonies were analyzed.

Forty-three mutants derived from J53(pMG252) were studied in detail. Ten mutants were selected at 0.5 $\mu\text{g}/\text{ml}$, 23 were selected at 0.87 $\mu\text{g}/\text{ml}$, and 10 were selected at 1 $\mu\text{g}/\text{ml}$ ciprofloxacin. There were no surviving colonies at higher ciprofloxacin concentrations.

TABLE 1 Ciprofloxacin, nalidixic acid, and novobiocin MICs of *E. coli* J53(pMG252) mutants

Strain	Ciprofloxacin concn for selection (μg/ml)	MIC (μg/ml)		
		Ciprofloxacin	Nalidixic acid	Novobiocin
J53		0.016	8	160
J53(pMG252)		0.25	32	160
J53(pMG252) Cip ^r mutants ^a				
2	1	1.5	128	1,280
3	1	2	128	1,280
4	1	2	64	1,280
5	1	2	128	1,280
6	1	2	8	40
7	1	0.87	128	320
8	1	0.5	64	320
9	1	1.5	64	640
10	1	0.75	64	1,280
11	1	1	128	160
12	0.87	2	128	1,280
13	0.87	2	128	1,280
14	0.87	2	128	1,280
15	0.87	0.87	8	10
16	0.87	0.87	8	20
17	0.87	0.87	128	1,280
18	0.87	0.75	8	20
19	0.87	1	8	20
20	0.87	0.87	8	10
21	0.5	0.87	32	80
22	0.5	1	32	160
23	0.5	0.87	32	5
24	0.5	1.5	64	1,280
25	0.5	1.5	64	1,280
26	0.5	2	32	160
27	0.5	1	64	320
28	0.5	2	128	1,280
29	0.5	0.87	8	20
30	0.5	0.87	32	1,280
2-1	0.87	1	32	640
2-2	0.87	2	64	640
3-1	0.87	1	64	20
3-2	0.87	2	64	40
4-1	0.87	1	64	640
5-1	0.87	1	128	640
5-2	0.87	1	64	1,280
6-1	0.87	1	64	640
8-1	0.87	2	32	640
8-2	0.87	2	64	320
9-1	0.87	0.5	64	320
9-2	0.87	2	64	5
10-1	0.87	1	64	1,280
10-2	0.87	2	64	1,280

^a Mutants 2 to 30 and 2-1 to 10-2 were selected in separate experiments. Pairs like 2-1 and 2-2 came from the same selection plate.

Susceptibility. Table 1 shows that the increases in MICs for the J53(pMG252) ciprofloxacin-resistant mutants ranged from 2- to 8-fold. The ciprofloxacin MBC for J53(pMG252) was 2 times the MIC, and this MBC/MIC ratio was unchanged in several ciprofloxacin-resistant mutants (data not shown). The nalidixic acid MIC was unchanged or increased up to 4-fold except in seven mutants, in which it decreased 4-fold.

In attempting to cure pMG252 from the mutants with novobiocin (14), we discovered that 12 of the mutants were hypersusceptible to novobiocin (including all those with increased nalidixic acid susceptibility), while 23 were ≥ 4 -fold more resistant than the parent (Table 1). Both ciprofloxacin and novobiocin target DNA gyrase but act on different subunits, and cross-resistance does not occur from target mutations. Consequently, decreased susceptibility to both ciprofloxacin and novobiocin suggested the activation of an efflux pump exporting both compounds.

Expression of efflux pumps. We sequenced the pump regulator genes *marR*, *robA*, *acrR*, *soxR*, and *soxS*; the *lon* protease gene, which degrades the products of some of these pump regulators; and *hns*, encoding a histone-like protein that regulates the expression of the *acrEF* and *mdtEF* drug exporter genes. We found that 11 mutants had alterations in MarR (Table 2), including one mutant with a 2-bp change (TA to AC) 22 bp upstream from the *marR* start codon at the binding site where MarR regulates its own expression (15). The *marR* mutations were mainly insertions and deletions causing frameshifts in the protein. In ciprofloxacin-resistant mutants 2 and 4, the same 47-bp duplication after bp 196 was found, creating a frameshift at amino acid 81. Mutant 6 had an A residue inserted at bp 126, creating a frameshift at amino acid 42. Four different deletions were observed: an A residue deleted at two different positions (bp 126 and bp 181) for mutants 12 and 20, a sizeable deletion of 144 bp in mutant 25, and a G deletion at bp 186 in mutant 27. Three strains had single point mutations, 2 of which had G→A transitions at bp 141, creating a TGA stop codon at amino acid 47. Mutations in *robA*, *acrR*, *lon*, *hns*, or *soxS* were not detected, but two mutants had G121D alterations in SoxR.

Inactivation of MarR increases the expression of *marA*, and with increased *marA* expression, the transcription of the pump genes *acrAB* and *tolC* is amplified, with consequently increased efflux of ciprofloxacin (16). To verify that MarR mutations caused a loss of repressor activity, we measured the expression levels of *marA* by RT-qPCR (Table 2) and found increased expression levels in the 11 strains containing MarR mutations. Similarly, expression of *soxS* is regulated by *soxR*, and increased *soxS* expression also leads to increases in *acrAB* transcript levels (17). We found 10- to 12-fold overexpression of *soxS* in the two strains with SoxR mutations (Table 2), thus establishing that in these mutants, SoxR was constitutively active. Finally, to establish whether the *acrAB* pump genes were overexpressed, we measured transcript levels of *acrA* in all 43 mutant strains by RT-qPCR. Eleven of the 13 MarR/SoxR mutant strains had 2- to 4-fold increases in the expression level of *acrA*, while the remaining 2 showed 1.39- to 1.75-fold increases (Table 2). *acrA* expression levels were not increased in any of the other mutants.

We also measured the relative expression levels of other efflux pumps that might contribute to ciprofloxacin resistance (Table 3). The *ydhE* efflux pump was overexpressed 2- to 3-fold in 6 mutants, and the *mdtEF* pump was overexpressed at least 2-fold in 9 mutants, in both cases independently of mutations in MarR or SoxR. Mutant 9-2 had a 2-fold increase in the expression level of *acrE*, and mutant 3-2, in addition to increased *ydhE* expression, also had a 2-fold increase in the expression level of *tolC*. Among the 9 mutants with overexpression of the *mdtEF* genes, mutants 15 and 28 showed 2-fold increases in the expression levels of the pump regulator *evgA*, the response regulator of the EvgAS two-component system (18). A 2- to 3-fold increase in the *evgA* expression level was also seen in mutants 2 and 4 containing MarR mu-

TABLE 2 Mutations in MarA and SoxS found in 13 J53(pMG252) Cip^r strains

J53(pMG252) Cip ^r strain	Mutated protein (mutation detected) ^a	Mean fold change in expression relative to J53(pMG252) (SEM)		
		<i>marA</i>	<i>soxS</i>	<i>acrA</i>
2	MarR (G81fs)	44.04 (11.49)	0.23 (0.07)	2.40 (0.91)
3	MarR (TA-AC) ^b	95.46 (19.40)	0.10 (0.04)	4.87 (1.54)
4	MarR (G81fs)	63.98 (24.51)	0.21 (0.12)	2.62 (0.62)
5	SoxR (G121D)	1.23 (0.11)	12.19 (3.22)	2.29 (0.48)
6	MarR (Q42fs)	35.74 (8.98)	0.33 (0.13)	2.16 (0.39)
12	MarR (N126fs)	36.36 (6.25)	0.311 (0.12)	2.22 (0.28)
13	SoxR (G121D)	1.73 (0.54)	10.55 (4.06)	2.51 (0.88)
14	MarR (C47STOP)	63.91 (7.21)	0.23 (0.09)	1.39 (0.25)
20	MarR (K62fs)	66.41 (22.37)	0.21 (0.07)	2.88 (1.02)
24	MarR (C47STOP)	28.22 (4.81)	0.34 (0.14)	2.30 (0.99)
25	MarR (V65fs)	46.27 (14.21)	0.24 (0.09)	1.75 (0.37)
27	MarR (V63fs)	9.20 (2.90)	0.32 (0.16)	2.21 (0.64)
28	MarR (V21D)	79.73 (1.22)	0.29 (0.15)	2.86 (0.65)

^a fs, frameshift; STOP, stop codon.

^b Mutation detected in the MarR binding site (22 bp before the GTG start codon).

tations and lacking overexpression of the *mdtEF* pump. None of the strains with overexpression of the *mdtEF* pump contained mutations in the 798-bp-long promoter region of *gadE*, another *mdtEF* regulator (19, 20). No changes in *mdfA* or in the *fis* and *dsrA* (21) regulators of MdtEF expression were detected.

LPS defects. Since a change in pump substrate specificity could conceivably account for the combination of decreased ciprofloxacin but increased novobiocin susceptibility, we sequenced genes for efflux pumps, *acrAB*, *acrEF*, *acrD*, *acrS*, *mdtEF*, and *ydhE*, but found no alterations. A clue to novobiocin hypersensitivity came from whole-genome sequencing, which disclosed a mutation in *rfaE*. RfaE participates in the biosynthesis of the ADP-L-glycero-D-manno-heptose precursors used in the assembly of inner core LPS. *E. coli* mutants lacking heptose in the LPS core display a variety of phenotypes due to the reduced stability of the outer membrane, including hypersensitivity to hydrophobic antibiotics such as novobiocin (22). Further sequencing (see Table 5) re-

vealed two additional mutants with defects in *rfaE* and two strains with mutations in *rfaD*. All of the *rfaD* and *rdjE* mutants were novobiocin hypersusceptible, but we were unable to detect a defect in six other novobiocin-hypersusceptible mutants despite sequencing 18 genes involved in LPS biosynthesis.

Porin expression. The major outer membrane proteins of *E. coli*, OmpF and OmpC, also have a role in susceptibility since they provide channels for ciprofloxacin entry and, when downregulated, decrease ciprofloxacin susceptibility (23, 24). Expression of the *ompC* and *ompF* porin genes was evaluated in all the mutants (Table 4). *ompF* expression was reduced 25% or more in 30 of the 43 mutants, while *ompC* expression was reduced by the same amount in 15 mutants. All but one of the mutants with *marR* or *soxR* defects had decreased *ompF* expression, averaging a decrement of >80%. In 9 of the 12 novobiocin-hypersusceptible mutants, *ompF* expression was reduced by at least 25%, averaging a decrease of 68%.

TABLE 3 Relative expression levels of other efflux pumps and regulators^a

Strain	Mean fold change relative to J53(pMG252) (SEM)						
	<i>mdfA</i>	<i>ydhE</i>	<i>acrE</i>	<i>tolC</i>	<i>mdtE</i>	<i>mdtF</i>	<i>evgA</i>
2-1	0.87 (0.25)	2.58 (0.82)	1.15 (0.44)	1.37 (0.15)	0.59 (0.19)	0.84 (0.30)	
2-2	0.88 (0.23)	3.15 (0.93)	1.35 (0.54)	1.29 (0.26)	0.62 (0.12)	0.78 (0.18)	
3-1	1.23 (0.35)	2.70 (0.31)	0.89 (0.43)	1.18 (0.14)	0.32 (0.08)	0.52 (0.05)	
3-2	0.65 (0.05)	2.17 (0.47)	1.43 (0.59)	2.02 (0.38)	1.18 (0.53)	1.46 (0.68)	
6-1	0.73 (0.29)	2.12 (0.42)	1.18 (0.34)	1.03 (0.11)	0.82 (0.29)	1.06 (0.32)	
8-1	1.23 (0.42)	2.10 (0.30)	0.98 (0.25)	0.86 (0.23)	0.35 (0.08)	0.61 (0.22)	
9-2	1.13 (0.31)	1.84 (0.79)	2.01 (0.80)	1.56 (0.25)	0.94 (0.15)	1.34 (0.18)	
2		1.34 (0.36)			0.94 (0.18)	1.03 (0.24)	2.04 (0.29)
4		1.70 (0.55)			1.71 (0.22)	1.40 (0.44)	2.40 (0.73)
12		0.92 (0.25)			2.11 (0.39)	2.78 (1.50)	1.25 (0.30)
13		1.61 (0.34)			2.10 (0.82)	3.76 (2.24)	1.44 (0.27)
15		0.78 (0.13)			2.68 (0.71)	1.66 (0.84)	2.10 (0.45)
21		0.55 (0.11)			2.10 (0.56)	1.52 (0.42)	1.14 (0.22)
24		1.52 (0.91)			2.78 (0.88)	2.01 (0.46)	1.33 (0.31)
25		0.83 (0.08)			2.43 (1.03)	2.22 (0.48)	1.42 (0.15)
26		0.57 (0.29)			3.04 (1.28)	1.58 (0.47)	1.37 (0.26)
28		1.52 (0.60)			2.50 (0.56)	2.38 (0.80)	2.44 (0.40)
30		0.73 (0.50)			6.96 (2.71)	5.49 (2.23)	0.96 (0.44)

^a At least three different assays with three different RNA extractions were performed for each gene tested.

TABLE 4 Relative expression levels of porins

Strain	Gene or promoter affected ^a	Mean fold change relative to J53(pMG252) (SEM)	
		<i>ompC</i>	<i>ompF</i>
J53(pMG252)	None	1.0	1.0
J53(pMG252) Cip ^r mutants			
2	<i>marR</i>	1.688 (0.575)	0.240 (0.044)
3	<i>marR</i>	1.612 (0.452)	0.168 (0.081)
4	<i>marR</i>	1.681 (0.406)	0.166 (0.051)
5	<i>soxR</i>	1.059 (0.291)	0.243 (0.107)
6	<i>marR</i>	1.078 (0.189)	0.127 (0.036)
7	—	2.381 (0.140)	1.312 (0.120)
8	<i>qnrA</i> ↑	2.702 (0.474)	0.900 (0.303)
9	<i>rpoB</i>	1.181 (0.182)	0.234 (0.006)
10	—	0.624 (0.213)	0.782 (0.061)
11	—	1.574 (0.468)	0.240 (0.027)
12	<i>marR</i> , <i>mdtE</i> ↑	1.379 (0.279)	0.199 (0.088)
13	<i>soxR</i> , <i>mdtE</i> ↑	1.298 (0.458)	0.281 (0.100)
14	<i>marR</i>	0.873 (0.206)	0.134 (0.015)
15	<i>mdtE</i> ↑	1.074 (0.088)	0.342 (0.030)
16	<i>rfaE</i>	0.972 (0.113)	0.271 (0.019)
17	<i>icdA</i>	0.907 (0.158)	1.287 (0.443)
18	<i>rfaD</i>	0.700 (0.075)	0.274 (0.036)
19	<i>rfaD</i> , <i>qnrA</i> ↑	0.862 (0.052)	0.350 (0.042)
20	<i>marR</i> , <i>rfaE</i>	0.831 (0.392)	0.127 (0.044)
21	<i>rfaE</i> , <i>mdtE</i> ↑	0.781 (0.326)	0.295 (0.030)
22	—	1.191 (0.112)	1.278 (0.239)
23	—	0.465 (0.129)	0.632 (0.072)
24	<i>marR</i> , <i>mdtE</i> ↑	1.756 (0.663)	0.206 (0.016)
25	<i>marR</i> , <i>mdtE</i> ↑	1.525 (0.290)	0.202 (0.067)
26	<i>mdtE</i> ↑	0.494 (0.106)	0.934 (0.326)
27	<i>marR</i>	1.862 (1.214)	0.994 (0.432)
28	<i>marR</i> , <i>mdtE</i> ↑	0.879 (0.280)	0.156 (0.027)
29	—	1.984 (0.426)	0.413 (0.082)
30	<i>mdtE</i> ↑	5.367 (1.882)	0.529 (0.026)
2-1	<i>ydhe</i> ↑	0.757 (0.207)	1.091 (0.268)
2-2	<i>ygeG</i> , <i>ydhe</i> ↑	0.572 (0.109)	1.064 (0.126)
3-1	<i>ydhe</i> ↑	0.372 (0.053)	0.951 (0.278)
3-2	<i>tolC</i> ↑, <i>ydhe</i> ↑	0.575 (0.264)	0.515 (0.081)
4-1	—	0.404 (0.026)	0.631 (0.147)
5-1	<i>pyeA</i>	0.764 (0.110)	0.886 (0.221)
5-2	<i>pyeA</i> , <i>yoaA</i>	0.375 (0.088)	1.087 (0.353)
6-1	<i>ydhe</i> ↑	0.313 (0.070)	0.801 (0.074)
8-1	<i>gudD</i> , <i>ydhe</i> ↑	0.271 (0.040)	0.458 (0.131)
8-2	<i>psdhC</i> , <i>qnrA</i> ↑	0.642 (0.251)	0.425 (0.133)
9-1	<i>acrE</i> ↑	0.329 (0.018)	0.707 (0.183)
9-2	—	0.382 (0.038)	1.146 (0.365)
10-1	—	0.278 (0.082)	0.351 (0.055)
10-2	—	0.211 (0.030)	0.679 (0.216)

^a —, no mutation was detected. The up arrow indicates increased.

Other chromosomal mutations. Mutations in eight other genes were detected in the remaining mutants but have not been proven to be responsible for the observed decrease in ciprofloxacin susceptibility (Table 5). *IcdA* is isocitrate dehydrogenase, a component of the tricarboxylic acid cycle. Defects in *icdA* have been associated with decreased nalidixic acid and ciprofloxacin susceptibilities via metabolic *acrA* activation (25), but *acrA* expression was not increased in our mutant. *RpoB* is the β -subunit of RNA polymerase. A mutation in the α -subunit (but not the

TABLE 5 Other mutations detected in J53(pMG252) Cip^r strains

Protein	Mutation detected	J53(pMG252) Cip ^r strain(s)
RfaE	Δ IS10	16
	H356L	20
	M217fs	21
RfaD	Δ IS10	18, 19
IcdA	Δ IS10	17
RpoB	R1246C	9
GudD	R213fs	8-1
YgeG	Y62H	2-2
YoaA	F308LLLLDdel	5-2
pYejA ^a	−4 G→A	5-1, 5-2
pSdhC ^a	−156 C→T	8-2
Protein of unknown function under GenBank accession no. EIE38512	G27fs	2-1, 2-2, 3-2, 8-1, 8-2, 10-1, 10-2

^a Promoter changes.

β -subunit) has been associated with decreased ciprofloxacin susceptibility in *Salmonella enterica* (26). *GudD* is glucarate dehydrogenase. *YgeG* is a putative secretion chaperone and a remnant of a presumably nonfunctional pathogenicity island. *YoaA* is a protein of unknown function with a nucleoside triphosphate hydrolysis domain. *YejA* is a component of a periplasmic peptide ABC transporter. *SdhC* is a membrane protein component of succinate:quinone oxidoreductase. The alterations in *YejA* and *SdhC* were located in their promoter regions. The mutant promoter from *SdhC* had 40% reduced activity when cloned upstream of *lacZ* in plasmid pRS415. In the same way, the mutant promoter from *YejA* showed 20% reduced activity. Finally, seven mutants obtained in the same experiment were defective in a 43-amino-acid protein of unknown function encoded by several *E. coli* genomes (GenBank accession number [EIE38512](#)). Further studies are needed to evaluate the role of these genes in resistance.

Changes in plasmid pMG252. Expression of *qnrA* is not regulated in pMG252, but the gene is surrounded by two copies of *ISCR1* with the potential for transposition to the chromosome or another site on the plasmid. *qnrA* expression was measured in all the mutants by RT-qPCR. Its expression levels were 2.4-fold higher in mutant 8, 2.2-fold higher in mutant 8-2, and 3.6-fold higher in mutant 19. No mutations were detected in the corresponding *qnrA* promoter regions. Mutants 8, 8-2, and 19 also had 1.9-fold, 3.1-fold, and 2.4-fold increases in *qnrA* copy numbers without a change in copy numbers for *sull1* or *bla*_{FOX-5}, two other resistance genes on pMG252, indicating that the *qnrA* copy number increase was gene specific. When pMG252 was displaced from J53(pMG252) mutants 8, 8-2, and 19 by selection with tetracycline for the entry of plasmid R692, belonging to the same Inc group as pMG252, ciprofloxacin susceptibility returned to the baseline value, supporting that a change in pMG252 is responsible for enhanced resistance. When plasmid pMG252 was outcrossed from these strains to *E. coli* C600, the level of ciprofloxacin resistance was 2- to 4-fold higher than that with unmodified pMG252. Furthermore, the copy number and expression level of *qnrA* in C600(pMG252) from mutants 8, 8-2, and 19 were also increased >3-fold. Although the plasmids have not been mapped, these changes suggest that *qnrA* duplication on pMG252 is the mechanism for decreased ciprofloxacin susceptibility in J53(pMG252) Cip^r mutants 8, 8-2, and 19.

TABLE 6 Characterization, susceptibility, and porin gene expression of *E. coli* J53 ciprofloxacin-resistant mutants

Strain	Ciprofloxacin concn for selection (µg/ml)	Mutation detected	Mean fold change relative to J53 (SEM) ^a		MIC (µg/ml) ^b						
			<i>ompC</i>	<i>ompF</i>	Cip	Nal	Novo	Amp	Chl	Ery	Trim
J53			1	1	0.016	8	640	8	8	128	0.25
Cip ^r mutants											
7	0.01	<i>mdfA</i> ↑	0.979 (0.132)	2.107 (0.195)	0.064	8	640	8	8	128	0.25
8	0.01		0.765 (0.272)	1.326 (0.070)	0.064	8	640	8	8	64	0.25
9	0.01	RfaE	0.310 (0.082)	0.213 (0.036)	0.032	1	20	8	2	8	0.125
10	0.01		0.606 (0.240)	1.370 (0.188)	0.064	8	640	8	8	128	0.25
11	0.01		0.627 (0.141)	1.170 (0.011)	0.064	8	640	8	8	128	0.5
12	0.01		0.851 (0.138)	0.231 (0.033)	0.064	8	640	16	16	128	0.25
13	0.02	LpcA	0.166 (0.053)	0.362 (0.016)	0.064	8	40	8	2	8	0.125
14	0.02	GyrA D391A ^c	0.534 (0.198)	0.900 (0.080)	0.064	1	20	8	4	32	0.25
15	0.02		0.479 (0.035)	0.348 (0.050)	0.064	32	640	16	8	128	4
16	0.02	SoxR	0.326 (0.141)	0.481 (0.165)	0.125	32	1280	16	32	128	2
17	0.02	SoxR	0.531 (0.192)	0.529 (0.137)	0.125	8	1280	16	32	128	2
18	0.02		0.497 (0.106)	0.252 (0.013)	0.064	1	80	16	32	64	0.25
19	0.04	MarR	0.474 (0.474)	0.091 (0.011)	0.25	32	1280	32	32	128	1
20	0.04	SoxR	0.312 (0.100)	0.268 (0.125)	0.25	32	2560	32	32	256	2
21	0.04		0.406 (0.051)	2.355 (0.270)	0.064	8	640	16	8	128	2
24	0.04	GyrA G81D	0.571 (0.288)	0.380 (0.023)	0.25	32	640	8	16	128	0.25
25	0.08	GyrA S83L	0.986 (0.284)	1.726 (0.147)	0.5	2048	640	8	8	128	0.5
26	0.08	GyrA S83L	0.847 (0.157)	1.504 (0.355)	1	2048	320	8	8	128	0.25
27	0.08	GyrA S83amber ^d	0.531 (0.216)	0.423 (0.065)	0.5	128	640	8	8	128	0.25

^a Values in boldface type differ by 50% or more from values for porin expression in the J53 parent.

^b MIC values in boldface type differ from values for the parent by at least 4-fold. Abbreviations: Cip, ciprofloxacin; Nal, nalidixic acid; Novo, novobiocin; Amp, ampicillin; Chl, chloramphenicol; Ery, erythromycin; Trim, trimethoprim.

^c Mutation outside the quinolone resistance-determining region.

^d Strain contains an amber suppressor.

Unexplained mutants. We have not yet been able to detect a chromosomal or plasmid alteration in 10 of the 43 mutants despite whole-genome sequencing. In particular, the entirety of the topoisomerase genes *gyrA*, *gyrB*, *parC*, and *parE* was examined in these strains, without discovering mutations within or outside the known QRDR.

Low-level ciprofloxacin resistance mutants of *E. coli* J53 without *qnrA*. To explore whether J53 without *qnrA* exposed to low concentrations of ciprofloxacin produced the same variety of mutations as seen with J53(pMG252), selection was performed with 0.01 to 0.16 µg/ml ciprofloxacin. Mutations in the QRDR of *gyrA* were present in 0 of 12 mutants selected with 0.01 or 0.02 µg/ml, 3 of 6 mutants selected with 0.04 µg/ml, and all 7 mutants selected with 0.08 or 0.16 µg/ml, including 1 with an amber mutation at serine 83 in GyrA. Such a mutant was reported previously (4) and is viable because of a coincident suppressor mutation (our unpublished observations). Nineteen mutants were analyzed in detail (Table 6). Ciprofloxacin MICs of the mutants varied from 0.032 to 1 µg/ml, and nalidixic acid MICs varied from 1 to 2,048 µg/ml, with high MICs resulting from *gyrA* mutations. Four strains were hypersensitive to novobiocin, while four had 2- to 4-fold increases in novobiocin resistance. Changes in susceptibility to ampicillin, chloramphenicol, erythromycin, and trimethoprim of 4-fold or higher were noted for some strains, as shown in Table 6. In 2 of the 4 novobiocin-hypersensitive strains, defects in core lipopolysaccharide biosynthesis were identified (I12L in *lpcA* and an IS10 insertion in *rfaE*). Five strains had mutations that increased efflux pump expression: 3 with R20C in SoxR and 14- to 26-fold increases in *soxS* expression, 1 in MarR

with K61stop and a 65-fold increase in *marA* expression, and 1 with a 2-fold increase in *mdfA* expression. Alterations in other genes, including *gyrB*, *parC*, and other genes of lipopolysaccharide biosynthesis, and increased expression of the pump gene *mdtE* or *yhde* were not detected. In 7 mutants with 4-fold increases in ciprofloxacin MICs, no mutation was found, indicating that the spectrum of low-level resistance mechanisms extends beyond those detected here.

DISCUSSION

The rarity of *gyrA* mutations in *E. coli* J53 carrying *qnrA* has been reported by several groups (4, 27). This result was initially unexpected because when *qnr* is added to a strain with a ciprofloxacin-resistant *gyrA* mutation, resistances are additive (28, 29), and both *gyrA* mutations and *qnr* genes are commonly found together in *E. coli* and other enterobacterial clinical isolates (30–32). Some combinations of *qnr* and *gyrA* mutants have a measureable decrease in growth rate (33), but the effect of the common GyrA S83L mutation and *qnrA* was an insignificant 1%, especially compared to the average 9% lower growth rate of the nalidixic acid-hypersusceptible mutants that we selected than of others (our unpublished observations), making fitness cost as an explanation for the lack of *gyrA* mutations unlikely. Mutations in *gyrA* were also rare in J53 carrying plasmids with enzymatic ciprofloxacin resistance [*aac(6′)-Ib-cr*] or a ciprofloxacin efflux pump (*qepA*), so the lack of *gyrA* mutations is not *qnr* specific. Furthermore, we have been unable to obtain *gyrA* mutants with secondary selection of higher ciprofloxacin resistance from J53(pMG252) Cip^r mutant 3 with a *marR* defect or Cip^r mutant 15 with defects in *rfaE* and increased

mdtE expression. Mutant selection in an *E. coli* strain carrying *qnr* thus provides a way to explore the range of nongyrase mutants that decrease ciprofloxacin susceptibility.

Mutations that are selected from J53(pMG252) with decreased ciprofloxacin susceptibility are mainly those that activate efflux pumps or alter LPS core biosynthesis. Mutations in MarR or SoxR that augment the expression of the major AcrAB/TolC pump were the most common, but increased expression of the MdtEF/TolC, YdhE, or AcrE efflux pump was found in other mutants. AcrAB is well known for ciprofloxacin efflux (34), and the potential for MdtEF (35), YdhE (36, 37), and AcrE (38) to efflux ciprofloxacin was also reported previously. Mutations upregulating drug efflux may have a fitness cost (39) but are relevant to resistance evolution because of their pleiotropic effects on susceptibility to other agents and potential for occurrence at a rate higher than that required for specific changes in the QRDR of *gyrA*. Gyrase mutations that provide ciprofloxacin resistance are relatively rare events because a change to an alternate amino acid at a relatively small number of localized sites in DNA gyrase is required. Mutations in pump regulatory proteins like MarR are more common because insertion, deletions, and substitutions at many sites can block translation. Gyrase mutations provide higher-level resistance than pump or porin changes, but Qnr, by competing with ciprofloxacin for its target (40); AAC(6′)-Ib-cr, by modifying the drug (7); and QepA, by enhancing ciprofloxacin efflux (8), all decrease the effective concentration of ciprofloxacin so that more common if less potent mutations at sites other than *gyrA* can predominate.

Measuring susceptibility to nalidixic acid and novobiocin as well as ciprofloxacin proved to be a useful phenotypic screening test. Enhanced resistance to all three agents suggested efflux pump activation, while unchanged nalidixic acid susceptibility and novobiocin hypersusceptibility suggested a defect in the core LPS pathway. Defects in many genes have been reported to cause novobiocin hypersusceptibility, including *cls* (41), *djlA* (42), *dnaK* and *dnaJ* (43), *mukB* (44), and *surA* (45), but especially genes of LPS biosynthesis (46, 47). We detected defects in *rfaD* and *rfaE* after discovering that 12 of the 43 J53(pMG252) ciprofloxacin-resistant mutants were novobiocin hypersusceptible and also found an *lpcA* mutant among low-level-ciprofloxacin-resistant mutants of J53 without *qnrA*. Increased novobiocin susceptibility combined with decreased quinolone susceptibility has been reported for *E. coli* mutants selected with tigecycline that had defects in the core LPS genes *lpcA*, *rfaC*, *rfaE*, and *rfaF* (48). Defects in LPS reduce the outer membrane barrier to novobiocin entry (49). Why ciprofloxacin susceptibility decreases instead relates to porin regulation.

OmpF is the major channel for ciprofloxacin entry in *E. coli*, with OmpC playing a lesser role (49, 50). Decreased expression levels of both *ompF* and *ompC* were detected in many of the ciprofloxacin-resistant mutants. These genes are transcriptionally regulated by the two-component systems OmpR-EnvZ (51) and CpxA-CpxR (52) in response to effects on the physical properties of the cell membrane due to such conditions as temperature, osmolarity, pH, and chemical stress (53). Each porin is also subject to posttranscriptional control by the small regulatory RNA molecules *micF* and *micC* that downregulate OmpF and OmpC expression (54, 55). Both MarA and SoxS activate *micF* production, explaining the decrease in *ompF* expression in MarR or SoxR mutants. We hypothesize that membrane stress from the mutant cell wall decreases porin expression in LPS mutants and explains

their decreased ciprofloxacin susceptibility. These changes in pump and porin expression also affect susceptibility to other antimicrobials, as is evident from changes in ampicillin, chloramphenicol, erythromycin, and trimethoprim susceptibilities with mutations providing low-level changes in ciprofloxacin susceptibility in *E. coli* J53 (Table 6).

In three strains, a change in the expression of *qnrA1* itself in pMG252 seemed to be responsible for enhanced resistance. *qnrA1* is bracketed by two copies of *ISCR1* (6), which has the potential to transpose to other sites on pMG252 or the *E. coli* chromosome. The increased copy number and expression of *qnrA* in these mutants as well as their transconjugants suggest that an increased gene dosage from replication on pMG252 is the mechanism for their reduced ciprofloxacin susceptibility.

Whether the other gene alterations that we discovered (Table 5) contribute to ciprofloxacin resistance requires further study. Quinolones are mutagenic and activate the cell's SOS system. At least 12 of the analyzed mutants had more than a single defect detected, so random mutations unrelated to ciprofloxacin resistance need to be excluded.

Low-level ciprofloxacin resistance can thus arise by many mechanisms, some of which increase or decrease susceptibility to chemically unrelated antibiotics. By decreasing the effective selecting ciprofloxacin concentration, *qnr* and other PMQR genes facilitate the selection of alterations in pumps, porins, and LPS, with multiple consequences for antimicrobial susceptibility.

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