ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Mar. 2011, p. 1266–1269 Vol. 55, No. 3
0066-4804/11/$12.00 doi:10.1128/AAC.00927-10

In Vitro Effect of *qnrA1*, *qnrB1*, and *qnrS1* Genes on Fluoroquinolone Activity against Isogenic *Escherichia coli* Isolates with Mutations in *gyrA* and *parC*▽

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Received 8 July 2010/Returned for modification 8 October 2010/Accepted 14 December 2010

This article provides an analysis of the *in vitro* effect of *qnrA1*, *qnrB1*, and *qnrS1* genes, combined with quinolone-resistant Ser83Leu substitutions in GyrA and/or Ser80Arg in ParC, on fluoroquinolone (FQ) resistance in isogenic *Escherichia coli* strains. The association of Ser83Leu substitution in GyrA, Ser80Arg substitution in ParC, and *qnr* gene expression increased the MIC of ciprofloxacin to 2 μg/ml. *qnr* genes present in *E. coli* that harbored a Ser83Leu substitution in GyrA increased mutant prevention concentration (MPC) values to 8 to 32 μg/ml. *qnr* gene expression in *E. coli* may play an important role in selecting for one-step FQ-resistant mutants.

Fluoroquinolone (FQ) resistance occurs mainly as a result of mutations in chromosomal genes encoding quinolone targets, DNA gyrase and topoisomerase IV (5). More recently, plasmid-mediated mechanisms, such as those mediated by the *qnr*, *aac*(*6′*)*-Ib-cr*, and *qepA* genes, have been reported (11, 18). In the absence of other mechanisms, the presence of any *qnr* gene increased the MIC of FQ between 4- and 128-fold, although MIC values remained below CLSI breakpoints (9, 18).

It has been suggested that Qnr proteins facilitate the selection of higher-level quinolone-resistant mutants. In spite of this, the therapeutic relevance of the acquisition of *qnr* genes on FQ bactericidal activity remains unclear (10, 13, 16). Since spontaneous bacterial mutants usually arise at a low frequency of 10^{-6} to 10^{-8}, the prevention of mutant bacterial populations may help to restrict the development of antimicrobial resistance. To avoid selecting for resistance, drug concentrations should be kept above the mutant prevention concentration (MPC) (4, 20). *In vivo* studies have shown that the presence of *qnr* genes in association with additional quinolone resistance mechanisms might be relevant in the activity of these antimicrobial agents (1, 15).

In a recent study (12), the combined effect of topoisomerase mutations on FQ resistance in isogenic *Escherichia coli* strains showed that at least three mutations—two of which had to be in *gyrA*—were necessary to exceed CLSI resistance breakpoints. Plasmid-mediated quinolone resistance (PMQR) genes confer low levels of quinolone resistance, and their precise effect on selecting for quinolone resistance in association with other mechanisms is not well known. In addition, recent studies have shown that the *qnrA* gene increased the MPC against FQ (16). The aim of this study was to evaluate the effect of *qnrA*, *qnrB*, and *qnrS* genes on the development of quinolone resistance in wild-type *E. coli* strains compared to isogenic *E. coli* strains harboring mutated *gyrA* and/or *parC* genes.

Ser83Leu and Ser80Arg mutations, located in GyrA and ParC, respectively, were obtained by gene replacement, as described by Posfai et al. (14). The *qnr* genes carried on the pBK-CMV cloning vector were transformed by electroporation into *E. coli* ATCC 25922 and its isogenic mutant strains *E. coli* ATCC 25922-S83L, *E. coli* ATCC 25922-S80R, and *E. coli* ATCC 25922-S83L-S80R (Table 1). The primers used to obtain the different isogenic strains are indicated in Table 2.

Susceptibility tests were performed in duplicate for each bacterial strain by the broth microdilution method according to CLSI reference methods (2). The presence of any *qnr* gene increased MIC levels in all *E. coli* genotypes. The ciprofloxacin (CIP) MIC for *E. coli* ATCC 25922 harboring any *qnr* gene was 0.125 μg/ml, which is more than 62-fold higher than that for the empty wild-type strain (Table 1). The expression of *qnr* genes in the *E. coli* ATCC-Ser83Leu strain gave a less marked increase, with CIP MICs of 0.5, 0.5, and 1 μg/ml, meaning 4-, 4-, and 8-fold increases in expression for *qnrA1*, *qnrB1*, and *qnrS1*, respectively. The Ser80Arg substitution in ParC played a secondary role in FQ resistance (Table 1), as previously described (7, 12). In *E. coli* ATCC-Ser83Leu-Ser80Arg, the presence and expression of *qnrA1* or *qnrS1* genes increased the CIP MIC to 2 μg/ml (or intermediate susceptibility according to CLSI guidelines) (Table 1) (2). Isogenic strains containing the *qnrB1* gene were always susceptible to FQ according to CLSI breakpoints, including the double-topoisomerase mutant (Table 1). *qnrB1* seems to be slightly less efficient than *qnrA1* and *qnrS1* in terms of MIC values. Minimal bactericidal concentrations (MBCs) were similar to the corresponding MIC values.

MPC was determined as described previously by Marcusson

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▽ Published ahead of print on 20 December 2010.

TABLE 1. Bacterial strains and fluoroquinolone MIC and MBCs

E. coli strain/ plasmid gene	Relevant features	Plasmid containing PMQR[a] gene	Ciprofloxacin susceptibility[b]	MIC (μg/ml)[c]				MBC (μg/ml)			
				CIP	LVX	MXF	NFX	CIP	LVX	MXF	NFX
ATCC 25922	Wild type	None	S	0.002	0.008	0.008	0.015	0.015	0.03	0.015	0.03
ATCC/*qnrA*	Wild type	pBK-QnrA1	S	0.125	0.5	0.25	0.5	0.125	0.5	0.5	1
ATCC/*qnrB*	Wild type	pBK-QnrB1	S	0.125	0.125	0.25	0.25	0.125	0.25	1	0.5
ATCC/*qnrS*	Wild type	pBK-QnrS1	S	0.125	0.5	0.25	0.5	0.25	0.5	0.25	1
ATCC 25922-S83L	GyrA Ser83Leu	None	S	0.125	0.125	0.06	0.125	0.125	0.25	0.125	0.5
ATCC-S83L/*qnrA*	GyrA Ser83Leu	pBK-QnrA1	S	0.5	0.5	0.5	2	0.5	0.5	0.5	2
ATCC-S83L/*qnrB*	GyrA Ser83Leu	pBK-QnrB1	S	0.5	0.25	0.5	1	0.5	0.5	0.5	1
ATCC-S83L/*qnrS*	GyrA Ser83Leu	pBK-QnrS1	S	1	1	1	2	1	2	2	4
ATCC 25922-S80R	ParC Ser80Arg	None	S	0.004	0.008	0.008	0.03	0.004	0.008	0.008	0.03
ATCC-S80R/*qnrA*	ParC Ser80Arg	pBK-QnrA1	S	0.25	0.25	0.5	0.5	0.25	0.5	0.5	2
ATCC-S80R/*qnrB*	ParC Ser80Arg	pBK-QnrB1	S	0.125	0.25	0.5	0.5	0.25	0.5	0.5	0.5
ATCC-S80R/*qnrS*	ParC Ser80Arg	pBK-QnrS1	S	0.125	0.25	0.25	0.5	0.25	0.25	0.5	0.5
ATCC 25922-S83L-S80R	GyrA Ser83Leu, ParC Ser80Arg	None	S	0.25	0.25	0.25	2	2	0.25	0.25	2
ATCC-S83L-S80R/*qnrA*	GyrA Ser83Leu, ParC Ser80Arg	pBK-QnrA1	I	2	2	2	8	4	4	4	64
ATCC-S83L-S80R/*qnrB*	GyrA Ser83Leu, ParC Ser80Arg	pBK-QnrB1	S	1	1	1	4	2	1	2	4
ATCC-S83L-S80R/*qnrS*	GyrA Ser83Leu, ParC Ser80Arg	pBK-QnrS1	I	2	4	2	8	4	4	4	16

[a] PMQR, plasmid-mediated quinolone resistance.
[b] S and I, susceptible and intermediate susceptibility, respectively, according to CLSI guidelines (2).
[c] MICs determined by microdilution for ciprofloxacin (CIP), levofloxacin (LVX), moxifloxacin (MXF), and norfloxacin (NFX).

et al. (8, 16). The presence of *qnr* genes increased MPC values of FQ in all *E. coli* genotypes. The MPC values of CIP in the wild-type *E. coli* ATCC 25922 strain coding for Qnr proteins as the only quinolone resistance mechanism increased 8-, 2-, and 4-fold for *qnrA1*, *qnrB1*, and *qnrS1*, respectively, compared to *E. coli* ATCC 25922 (Table 3). A similar increase was observed for the other quinolones. The presence of a single Ser83Leu mutation in the *gyrA* gene raised MPC values 2- to 4-fold, compared to those for wild-type *E. coli* ATCC 25922. The additional presence of *qnr* genes in *E. coli* ATCC-Ser83Leu increased MPC values to 8 to 32 μg/ml, depending on the FQ (Table 3). *E. coli* ATCC-Ser83Leu-Ser80Arg MPC values ranged from 4 to 32 μg/ml. *E. coli* ATCC-Ser83Leu-Ser80Arg expressing *qnr* genes showed MPC values ranging from 32 to 128 μg/ml (except for *qnrB1*, for which values ranged from 8 to 32 μg/ml), well in excess of the breakpoint concentrations of CLSI guidelines (data not shown). The effect of *qnr* genes on MPC was similar to the presence of a Ser83Leu substitution in GyrA as the single quinolone resistance mechanism. MPC concentrations were clearly higher than the maximum serum concentrations obtained when using drugs in antimicrobial therapy for *E. coli* ATCC 25922-Ser83Leu expressing *qnr* genes (6, 19). The presence of quinolone resistance mechanisms produced a reduction in the mutant selection window (MSW). It is therefore difficult to predict MPC from the MIC values, and on this basis, the MPC will vary according to FQ and the specific resistance mechanism involved (Table 3).

Mutants were recovered from the plated concentrations closest to the MPC value at a very low frequency. The quinolone resistance-determining region (QRDR) of target genes *gyrA* and *parC* was analyzed. All of the characterized mutants of the *E. coli* ATCC 25922 strain had just a Ser83Leu substitution in the QRDR of *gyrA*, supporting the view that this is the most frequent modification in *E. coli*. On the other hand, most colonies of the *E. coli* ATCC-Ser83Leu strain selected in the MSW showed additional modifications in the QRDR of the *parC* gene (Gly78Asp or Ser80Ile substitutions), also previously associated with quinolone resistance. Clinical FQ resistance according to CLSI guidelines (2) (MIC of ≥4 μg/ml for CIP) was observed for some of these mutants (Table 3). The PMQR might enable mutant bacteria with low levels of FQ resistance to survive long enough for them to grow and emerge during FQ treatment. The detection of mutations in type II topoisomerase genes reflects the ability of this mechanism to select for mutants with higher quinolone resistance. *In vivo* selection of FQ-resistant *Enterobacteriaceae* expressing *qnr* genes has been reported (3, 13). With respect to bacterial survival, although some bacteria did survive the MPC for a 96-h extended period, no quinolone-resistant mutants were selected, these being a persistent phenotype and indicating that the MPC parameter was working as specified (Table 3) (8).

Killing-curve assays showed a selective advantage for survival at 1 μg/ml of CIP in strains expressing *qnr* genes, both with and without the Ser83Leu substitution in GyrA in *E. coli* (Fig. 1). This CIP concentration defines the limit for establishing susceptibility or intermediate susceptibility (according to CLSI guidelines) in *Enterobacteriaceae* (2). CIP at 1 μg/ml in the isogenic wild-type *E. coli* strain ATCC 25922 (with or without *qnr* gene expression [Fig. 1A]) caused a marked re-

TABLE 2. Oligonucleotides and plasmids used in this study

Primer or plasmid	Sequence[a]	Use in this study	Source or reference
qnr cloning			
Pre-QnrA1	5′-CGGGATCCCGCGGCAGTTAAAATTGGGGCT-3′	Cloning of *qnrA1*	This study
Post-QnrA1	5′-CGGGATCCCGACGCCGAGTCCCGACCAGACTGC-3′	Cloning of *qnrA1*	This study
Pre-QnrB1	5′-CGGGATCCCGCTTGGTCGCCCTGGCCAACC-3′	Cloning of *qnrB1*	This study
Post-QnrB1	5′-CGGGATCCCGGCAAACCAGCTTACAGCAGGC-3′	Cloning of *qnrB1*	This study
Pre-QnrS1	5′-CGGGATCCCGCCACTTAAAACAGGTAAATTG-3′	Cloning of *qnrS1*	This study
Post-QnrS1	5′-CGGGATCCCGTACATGGTTGTCCCTATGTC-3′	Cloning of *qnrS1*	This study
Gene replacement			
gyrAS83L-Fw	5′-CCATGGTGACCTGGCGGTCTATG-3′	Mutagenesis of *gyrA*	This study
gyrAS83L-Rv	5′-CATAGACCGCCAGGTCACCATGG-3′	Mutagenesis of *gyrA*	This study
parCS80R-Fw	5′-CCGCACGGCGATCGCGCCTGTTATGAAGC-3′	Mutagenesis of *parC*	This study
parCS80R-Rv	5′-GCTTCATAACAGGCGCGATCGCCGTGCGG-3′	Mutagenesis of *parC*	This study
Pre-gyrAS83	5′-CGGGATCCCGAGCGATCTCTTCGTGGTCTACG-3′	Partial *gyrA* amplification	This study
Post-gyrAS83	5′-CGGGATCCCGCCTGATACGGAATTTCGTGGAC-3′	Partial *gyrA* amplification	This study
Pre-parCS80	5′-CGGGATCCCGGACCGCGATAGCGTTGTCTTCCG-3′	Partial *parC* amplification	This study
Post-parCS80	5′-CGGGATCCCGCAGATCGGTGGTAGCGAAGAGGTG-3′	Partial *parC* amplification	This study
QRDR[b] sequencing			
gyrA-1	5′-AAATCTGCCCGTGTCGTTGGT-3′	Sequencing	17
gyrA-2	5′-GCCATACCTACGGCGATACC-3′	Sequencing	17
parC-A	5′-CTGAATGCCAGCGCCAAATT-3′	Sequencing	17
parC-B	5′-GCGAACGATTTCGGATCGTC-3′	Sequencing	17
Plasmids			
pBK-CMV	Cloning vector		
pST76C	Gene replacement/suicide vector		
pUC19RP12	Gene replacement/resolution vector		

[a] Underlined nucleotides correspond to the BamHI site used for cloning.
[b] QRDR, quinolone resistance-determining region.

duction in viable bacteria after 8 h of incubation. After 6 h, no viable bacteria were recovered for the wild-type *E. coli* ATCC 25922 strain without *qnr* genes, while *E. coli* ATCC 25922 with *qnr* gene expression maintained levels of 10^2 to 10^3 CFU/ml for up to 24 h. At 4× the MIC of CIP, all *qnr* gene expression in wild-type *E. coli* ATCC 25922 maintained a viable CFU/ml at least 100-fold higher at 24 h compared to empty wild-type strains (data not shown). CIP at 1 μg/ml in the isogenic *E. coli* ATCC-Ser83Leu strain (with and without *qnr* gene expression [Fig. 1B]), caused a marked reduction in viable bacteria during the first 8 h, except for strains expressing *qnrS1*. After 8 h, bacterial regrowth was noted for strains expressing *qnrA1*, *qnrB1*, and *qnrS1*, and this continued up to 24 h, although not for *E. coli* ATCC-Ser83Leu, demonstrating the impact of *qnr* genes in terms of bacterial viability.

Finally, we evaluated 16 isogenic *E. coli* strains harboring different QRDR modifications, and with and without *qnr*-expressing genes such as *qnrA1*, *qnrB1*, and *qnrS1*. This study showed that these mechanisms, implicated in low-level plasmid-mediated FQ resistance, may play a significant role in the

TABLE 3. Fluoroquinolone MPC, MSW, MPC time window, and MIC or MIC range for mutants of the eight isogenic strains used in this study

E. coli strain/plasmid gene	MPC (μg/ml)[a]				MSW[b] (MPC/MIC [μg/ml])				MPC time window (h)[c]				MIC or MIC range for mutant (μg/ml)[d]			
	CIP	LVX	MXF	NFX	CIP	LVX	MXF	NFX	CIP	LVX	MXF	NFX	CIP	LVX	MXF	NFX
ATCC 25922	1	2	2	4	500	250	250	266.7	48	48	48	48	0.06	0.125	0.06	0.25
ATCC/*qnrA*	8	8	8	8	64	16	32	16	48	24	24	24	0.5	1–2	0.5–1	1–2
ATCC/*qnrB*	2	4	4	8	16	32	16	32	72	48	24	48	0.125	0.5	0.5–1	0.5–1
ATCC/*qnrS*	4	4	4	8	32	8	8	16	24	24	24	24	0.5–1	1–2	0.5–1	1–2
ATCC 25922-S83L	4	4	4	8	32	32	66.7	32	24	24	24	24	0.5	2	1	2
ATCC-S83L/*qnrA*	16	32	16	16	32	64	64	16	24	24	24	24	1–4	2–4	2–4	8–16
ATCC-S83L/*qnrB*	8	16	32	16	16	64	64	16	24	72	24	24	1–2	1–4	1–4	2–16
ATCC-S83L/*qnrS*	8	16	32	32	8	16	32	16	24	96	24	24	1–2	4	1–2	2–16

[a] MPC values were determined on Mueller-Hinton plates for ciprofloxacin (CIP), levofloxacin (LVX), moxifloxacin (MXF), and norfloxacin (NFX); MPC was defined as the lowest antibiotic concentration (in the range of concentration steps analyzed) at which resistant colonies do not form.
[b] MSW, mutant selection window (i.e., the antibiotic concentration found between the MIC and MPC).
[c] Earliest time (in hours) at which resistant colonies were visible one step below the MPC.
[d] MICs for resistant colonies were recovered on Mueller-Hinton plates one step below the MPC value.

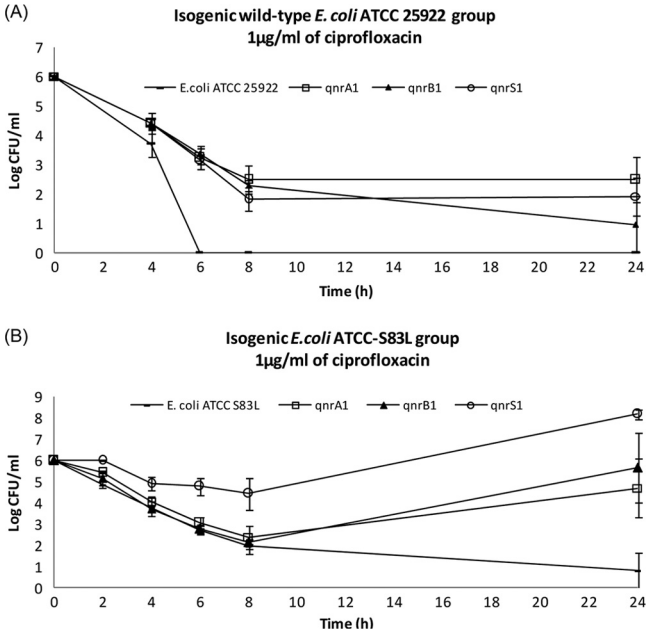

FIG. 1. Viable bacterial counts in time-kill curve assays with ciprofloxacin (CIP) 1 μg/ml. (A) Isogenic wild-type *E. coli* ATCC 25922, with and without *qnrA1*, *qnrB1*, or *qnrS1* gene expression; (B) isogenic mutant *E. coli* ATCC 25922-S83L, with and without *qnrA1*, *qnrB1*, or *qnrS1* gene expression.

acquisition of clinical resistance to FQ and, therefore, therapeutic failure. Animal models are necessary to confirm these *in vitro* results.

This work was supported by the Ministerio de Sanidad y Consumo, Instituto de Salud Carlos III (project PI060580) and the Consejería de Innovación Ciencia y Empresa, Junta de Andalucía (P07-CTS-02908), Spain. It was partly supported by the Ministerio de Sanidad y Consumo, Instituto de Salud Carlos III-FEDER, Spanish Network for Research in Infectious Diseases (REIPI RD06/0008). A.B. was funded by a predoctoral grant from the Instituto de Salud Carlos III (PFIS), Spain.

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