ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 2007, p. 4105–4110 Vol. 51, No. 11
0066-4804/07/$08.00+0 doi:10.1128/AAC.00616-07
Copyright © 2007, American Society for Microbiology. All Rights Reserved.

Prevalence and Expression of the Plasmid-Mediated Quinolone Resistance Determinant *qnrA1*▽

Xiaogang Xu,[1] Shi Wu,[1] Xinyu Ye,[1] Yang Liu,[1] Wanliang Shi,[1]
Yingyuan Zhang,[1] and Minggui Wang[1,2]*

Institute of Antibiotics, Huashan Hospital,[1] and Institute of Biomedical Sciences,[2] Fudan University, Shanghai 200040, People's Republic of China

Received 10 May 2007/Returned for modification 2 July 2007/Accepted 16 August 2007

Since its discovery, *qnrA* has been found in most common *Enterobacteriaceae*. Ciprofloxacin MICs conferred by different *qnrA*-positive plasmids could range from 0.1 μg/ml to 2 μg/ml in *Escherichia coli* J53. The reasons for different ciprofloxacin MICs conferred by *qnrA* have not been fully clarified. Five hundred forty-one consecutive gram-negative clinical strains that were resistant or intermediate to ciprofloxacin and that were isolated in Shanghai in 2005 were screened for *qnrA* by PCR. For *qnrA*-positive isolates, the transferability of quinolone resistance was determined by conjugation and mutations within the quinolone resistance-determining region (QRDR) of *gyrA* and *parC*. *aac(6′)-Ib-cr* was detected and *qnrA* RNA expression was determined using real-time reverse transcription-PCR for transconjugants with different ciprofloxacin MICs. The *qnrA* gene was detected in 7 of the 541 clinical isolates. Quinolone resistance was transferred in four strains by conjugation. Mutations in the QRDR of *gyrA* and *parC* were detected in five *qnrA*-positive clinical strains with higher ciprofloxacin MICs. Of four *qnrA*-bearing plasmids in *E. coli* J53, pHS4 and pHS5 conferred ciprofloxacin MICs of 0.094 to 0.125 μg/ml; pHS3, which harbored the *aac(6′)-Ib-cr* gene as well, conferred a ciprofloxacin MIC of 0.25 μg/ml, and pHS6, which had both the *aac(6′)-Ib-cr* gene and a high expression level of *qnrA*, had a ciprofloxacin MIC of 1.0 μg/ml. The prevalence of *qnrA* appeared to be higher in *Enterobacter cloacae* than in other *Enterobacteriaceae*. The coexistence of *qnrA* and *aac(6′)-Ib-cr* in a single plasmid and increased *qnrA* expression can account for the different levels of ciprofloxacin resistance seen in transconjugants.

Plasmid-mediated quinolone resistance associated with the *qnrA* gene was discovered in 1998 (10). The originally described *qnr* gene is now referred to as *qnrA* because of recent findings of other related *qnr* genes, *qnrB* (7) and *qnrS* (5). Since the discovery of *qnrA*, *qnrA* was found in almost all populated continents and in most common *Enterobacteriaceae* including *Escherichia coli*, *Klebsiella* spp., *Enterobacter* spp., *Citrobacter freundii*, and *Providencia stuartii* (6, 16). The prevalence of *qnrA* varied from less than 1% to higher than 20%, depending on the population sampled. Plasmids harboring *qnrA* may also encode an extended-spectrum β-lactamase (ESBL) (6). *qnrA* was absent in nonfermenting gram-negative bacteria such as *Pseudomonas aeruginosa* and *Acinetobacter* spp. in small surveys, but whether the lack of detection reflects a true absence or the small number of strains tested is not clear (16). Most of these studies screened for *qnrA* from clinical isolates collected in the late 1990s or early 2000s, several years prior to each study. Usually, the strains screened had additional restriction conditions such as resistance to expanded-spectrum cephalosporins or carriage of genes encoding ESBLs simultaneously. Consecutive strains of *Enterobacteriaceae* were screened for *qnrA* in a few surveys (1).

qnrA conferred a low level of quinolone resistance, and the clinical strains that harbored *qnrA* usually had much higher MICs of ciprofloxacin than their respective transconjugants. The *qnrA*-bearing clinical strains might have an active efflux mechanism, decreased permeability, and/or additional mutations in the genes encoding the subunits of DNA gyrase and topoisomerase IV (22). Another bacterial defense mechanism is *aac(6′)-Ib-cr*, a variant of aminoglycoside acetyltransferase. In one study of these plasmids carrying *qnrA*, it was found that *aac(6′)-Ib-cr* had the ability to N-acetylate ciprofloxacin and norfloxacin at the amino nitrogen on its piperazinyl substituent. Other quinolones lacking unsubstituted piperazinyl nitrogen were unaffected. When the *aac(6′)-Ib-cr* gene was cloned into pBC SK and introduced into *E. coli* DH10B cells, the MIC of ciprofloxacin increased threefold to fourfold, and *aac(6′)-Ib-cr* increased the frequency of selection of chromosomal mutants upon exposure to ciprofloxacin substantially (19).

In this study, we examined the presence of the plasmid-mediated quinolone resistance determinant *qnrA* in consecutive gram-negative clinical strains that were resistant or intermediate to ciprofloxacin, regardless of the susceptibility to other antimicrobials including cephalosporins, isolated in a teaching hospital of Fudan University in Shanghai from 15 February to 15 July 2005. We have found that the transcriptional level of the *qnrA* gene had substantial effects on the *qnrA* expression level and hence the resistance of the strain to ciprofloxacin. Furthermore, *aac(6′)-Ib-cr* was identified as coexisting with *qnrA* in two plasmids. Finally, we identified the mutations within the quinolone resistance-determining region (QRDR) of *gyrA* and *parC* associated with resistance to quinolones.

* Corresponding author. Mailing address: Institute of Antibiotics, Huashan Hospital, Fudan University, 12 M. Wulumuqi Rd., Shanghai 200040, People's Republic of China. Phone: (86-21)-62489999, ext. 6507. Fax: (86-21)-62488290. E-mail: mgwang@fudan.edu.cn.
▽ Published ahead of print on 27 August 2007.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or relevant characteristic(s)	Source or reference
Strains		
E. coli J53Az^R	Resistant to azide, *met pro*	10
E. coli HB101	F′ Δ(*gpt-proA*)*62 leuB6 supE44 ara-14 galK2 lacY1* Δ(*mcrC-mrr*) *xyl-5 mtl-1 recA13 rpsL20* (Str^r)	TaKaRa
E. coli V517	Multiple-plasmid-containing strain used as the molecular marker for plasmid	22
E. coli R1	*E. coli* J53 containing plasmid R1 (92 kb)	22
E. coli Plac	*E. coli* J53 containing plasmid Plac (152 kb)	22
E. coli R27	*E. coli* J53 containing plasmid R27 (182 kb)	22
UAB1	*K. pneumoniae* containing pMG252, a *qnrA*-bearing plasmid	10
Plasmids		
pKK232-8	Promoter selection vector, replicon pMB1, Ap^r	Pharmacia
pKK232-8-HS3	pKK232-8 containing the promoter for *qnrA* from plasmid pHS3	This study
pKK232-8-HS4	pKK232-8 containing the promoter for *qnrA* from plasmid pHS4	This study
pKK232-8-HS5	pKK232-8 containing the promoter for *qnrA* from plasmid pHS5	This study
pKK232-8-HS6	pKK232-8 containing the promoter for *qnrA* from plasmid pHS6	This study

MATERIALS AND METHODS

Bacterial strains and plasmids. Five hundred forty-one consecutive gram-negative clinical strains that were resistant or intermediate to ciprofloxacin were collected from a teaching hospital of Fudan University in Shanghai, China, between 15 February and 15 July 2005, including 169 strains of *K. pneumoniae*, 128 of *P. aeruginosa*, 98 of *E. coli*, 77 of *A. baumannii*, 13 of *Enterobacter* spp. (11 *Enterobacter cloacae* and 2 *Enterobacter aerogenes*), 9 of *Citrobacter* spp., 20 of other *Enterobacteriaceae* (6 of *Proteus mirabilis*, 6 of *Klebsiella oxytoca*, 5 of *Serratia marcescens*, and 3 of *Morganella morganii*), and 27 of other nonfermenting bacilli (11 strains of *Stenotrophomonas maltophilia*, 9 of *Flavobacterium* spp., 4 of *Alcaligenes* spp., and 3 of other *Acinetobacter* spp.). Inhibition zone diameter interpretive standards and MIC breakpoints for ciprofloxacin followed CLSI criteria: susceptible, zone diameter of ≥21 mm or MIC of ≤1 μg/ml; intermediate, zone diameter of 16 to 20 mm or MIC of 2 μg/ml; resistant, zone diameter of ≤15 mm or MIC of ≥4 μg/ml (2). Each isolate was from a separate patient, and most were from hospitalized patients. Additional strains and plasmids used in the study are given in Table 1.

Screening for the *qnrA* gene and conjugation. The strains were screened for the presence of the *qnrA* gene by PCR with primers qnrA F and qnrA R (Table 2) to produce a 627-bp amplification product as previously described (21). Both strands of purified PCR products of *qnrA* were sequenced using dye terminators with an ABI 3730 automated sequencer.

In order to determine if quinolone resistance was transferable in the strains with *qnrA*-bearing plasmids, conjugation experiments were carried out in LB broth with *E. coli* J53Az^R as the recipient as previously described (21). Transconjugants were selected on Trypticase soy agar plates containing sodium azide (100 μg/ml) for counterselection and sulfamethoxazole (300 μg/ml), cefotaxime (10 μg/ml), gentamicin (10 μg/ml), or chloramphenicol (50 μg/ml) to select for plasmid-encoded resistance.

Plasmid DNAs were isolated with the QIAGEN (Hilden, Germany) Plasmid Midi kit, and plasmid size was estimated as previously described (22).

MIC determination. MICs for the donor, recipient, transconjugant, and transformant strains were measured by agar dilution in accordance with the guidelines of the CLSI (2) for ciprofloxacin, ampicillin, cefotaxime, chloramphenicol, gentamicin, kanamycin, levofloxacin, streptomycin, sulfamethoxazole, tetracycline, tobramycin, and trimethoprim. The Etest (Biodisk AB, Solna, Sweden) was used to detect minimal changes in ciprofloxacin, levofloxacin, and chloramphenicol susceptibility.

QRDR sequencing. The PCR amplifications were carried out with the TaKaRa LA PCR kit, and incubation conditions were those suggested by the manufacturer. Primers (Table 2) were used to amplify *gyrA* and *parC*. Purified PCR products were sequenced on both strands, and QRDR DNA sequences of *gyrA* and *parC* for each of the *qnrA*-positive isolates were compared with the QRDR DNA sequences of *E. cloacae*, *E. coli*, and *C. freundii* (GenBank accession numbers were AF052256, NC000913, and AF052253 for *gyrA* and D88981, NC000913, and AB003914 for *parC*, respectively).

Detection of aminoglycoside acetyltransferase *aac(6′)-Ib*. The *aac(6′)-Ib* gene was PCR amplified with primers aac F and aac R (Table 2). PCR products were purified prior to sequencing on both strands.

ESBL genes (CTX-M, SHV, TEM, VEB, PER, and SFO) and plasmid-mediated AmpC β-lactamase genes were sought by PCR with specific primers (primers for the amplification of bla_{CTX-M} are shown in Table 2, and primers for AmpC genes were previously described by Perez-Perez and Hanson [14]). PCR products were sequenced on both strands.

RNA preparation and real-time RT-PCR. Total RNA was prepared using the TaKaRa RNAiso reagent, treated with DNase I, and purified according to the manufacturer's protocol. TaqMan reverse transcription reagents (Applied Biosystems) were used to synthesize cDNA from RNA samples. A total of 1.0 μg total RNA was used in a 50-μl reverse transcription (RT) reaction, and incubation conditions were those suggested by the manufacturer: 25°C for 10 min, 48°C for 30 min, and 95°C for 5 min. Following RT, 2 μl cDNA reaction products was used per 25 μl of real-time PCR employing SYBR green detection. All reactions

TABLE 2. Primers used in this study

Gene or plasmid and primer	Sequence	Source or reference
qnrA		
qnrA F[a]	5′-TCAGCAAGAGGATTTCTCA-3′	21
qnrA R[a]	5′-GGCAGCA CTATTACTCCCA-3′	21
RTqnrA F[b]	5′-TGCCAACTGCTTTGGCATAGA-3′	This study
RTqnrA R[b]	5′-TGGCCACTCAAGTTGGTATAGG-3′	This study
16S rRNA		
RT16S F[b]	5′-GCATAACGTCGCAAGACCAAAG-3′	This study
RT16S R[b]	5′-TTCTTCATACACGCGGCATGG-3′	This study
gyrA		
gyrA F[a]	5′-AAATCTGCCCGTGTCGTTGGT-3′	20
gyrA R[a]	5′-GCCATACCTACGGCGATACC-3′	20
parC		
parC F[a]	5′-CTGAATGCCAGCGCCAAATT-3′	20
parC R[a]	5′-GCGAACGATTTCGGATCGTC-3′	20
aac(6′)-Ib		
aac F[a]	5′-ATATGCGGATCCAATGAGCAACGCAA AAACAAAGTTAG-3′	19
aac R[a]	5′-ATATGCGAATTCTTAGGCATCACTGCG TGTTCGCTC-3′	19
bla_{CTX-M}		
CTX-M-G1-F	5′-AGT GCA AAC GGA TGA TGT-3′	This study
CTX-M-G1-R	5′-GGC TGG GTA AAA ATA GGT C-3′	This study
qnrA promoter		
Promoter F	5′-CCGGATCCCGACCCCAAATCCAACA-3′	This study
Promoter R	5′-GGGAAGCTTACGGCTTCCTTTAATCA G-3′	This study
pKK232-8		
pKK-SQ	5′-TCCGGATGAGCATTCATCAG-3′	This study

[a] Primers were used for amplification and sequencing.
[b] Primers were used to carry out real-time PCR.

TABLE 3. Characteristics of *qnrA*-positive clinical strains

Organism and strain	Ward of isolation	MIC (μg/ml)[a]				
		CIP	CTX	CAZ	GEN	SMZ
E. cloacae						
63	Geriatrics[b]	32	64	32	4	≥512
91	Neurosurgery[c]	4	32	128	8	≥512
113	Geriatrics[b]	128	64	128	8	≥512
641	Worldwide medical center[d]	4	32	64	4	≥512
E. coli						
633	Neurosurgery[c]	≥128	≥128	4	≥128	≥512
650	Geriatrics[b]	32	1	4	≥128	≥512
C. freundii						
64	Nephrology	32	2	4	32	≥512

[a] CIP, ciprofloxacin; CTX, cefotaxime; CAZ, ceftazidime; GEN, gentamicin; SMZ, sulfamethoxazole.
[b] These three strains were isolated from the same ward from March 7 to July 5.
[c] These two strains were isolated from two different wards of the neurosurgery department.
[d] This inpatient had peritonitis because of appendicitis with perforation.

were performed in tetraplicate. Amplification of an endogenous control, the 16S rRNA gene, was performed to standardize the amount of sample RNA or DNA added to a reaction. Primer sequences used for each gene target are presented in Table 2.

Real-time PCR was performed using SYBR green PCR master mix (Applied Biosystems) and carried out using an ABI Prism 7300 sequence detection system. PCR cycling conditions were 1 cycle at 50°C for 2 min, which was followed by 1 cycle at 95°C for 10 min, which was then followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Results were analyzed with the ABI Prism 7300 sequence detection system software. Relative quantification was determined by the $2^{-\Delta\Delta C_T}$ or $\Delta\Delta C_T$ method. Expression of the endogenous control gene, 16S rRNA, was used to normalize data.

Cloning of the *qnrA* promoters in pKK232-8. The promoter-like sequences of *qnrA* (9) and the common-region right-hand boundary were amplified with PCR by using primers promoter F, containing the BamHI site, and promoter R, containing the HindIII site, from *qnrA*-bearing plasmids pHS3, pHS4, pHS5, and pHS6. The PCR products with sizes of 274 bp or 281 bp were cut with BamHI plus HindIII, resulting in a 262-bp or 269-bp fragment, and cloned into pKK232-8, creating pKK232-8-HS3, pKK232-8-HS4, pKK232-8-HS5, and pKK232-8-HS6 (Table 1). All promoter fragments were sequenced using primer pKK-SQ after their final insertion into plasmids to verify that no mutations occurred during PCR or cloning. After cloning of the *qnrA* promoters into pKK232-8, these plasmids and pKK232-8 were transformed into HB101.

RESULTS

Screening for the *qnrA* gene and conjugation. The *qnrA* gene was detected in 7 of the 541 ciprofloxacin-resistant or -intermediate clinical strains consecutively isolated from a teaching hospital in Shanghai, China, including 4 of 13 (30.8%) *E. cloacae*, 2 of 98 (2.0%) *E. coli*, and 1 of 9 (11%) *Citrobacter* sp. strains. *qnrA* was not found in 169 strains of *K. pneumoniae* and 20 strains of other *Enterobacteriaceae*. All seven *qnrA*-positive strains, five strains isolated from sputum and one each from wound exudate and urine, were isolated from inpatients, including six patients with nosocomial infections and one patient with asymptomatic bacteriuria. These seven strains were isolated from five different wards (Table 3). Antimicrobial agents were used in six patients, and fluoroquinolones (ciprofloxacin or levofloxacin) were used in five patients before the isolation of *qnrA*-positive strains. The patient with bacteriuria had not been exposed to an antimicrobial agent prior to isolation of the *qnrA*-positive *C. freundii* isolate. *qnrA* was not found in any of the 128 strains of *P. aeruginosa*, 77 strains of *A. baumannii*, and 27 strains of other nonfermenting bacillus strains.

Out of seven *qnrA*-positive strains, four strains transferred quinolone resistance onto plasmids of different sizes by conjugation. Among the transferable plasmids, pHS3, pHS4, and pHS5 were greater than 150 kb in size, while pHS6 was approximately 65 kb. Each strain had a unique plasmid profile (Fig. 1 and 2). The MICs of ciprofloxacin against transconjugants demonstrated an 11-fold difference, from 0.094 μg/ml to 1 μg/ml, or were 12- to 125-fold higher than the recipient *E. coli* strain J53 (Table 4). Three other *qnrA*-bearing strains failed to produce transconjugants, although high-molecular-weight plasmids were visualized in donor strains (Fig. 1), and multiple agents were used for selection.

qnrA contained in all seven strains was identical to the *qnrA* sequence in the reported Shanghai *E. coli* strains (22)

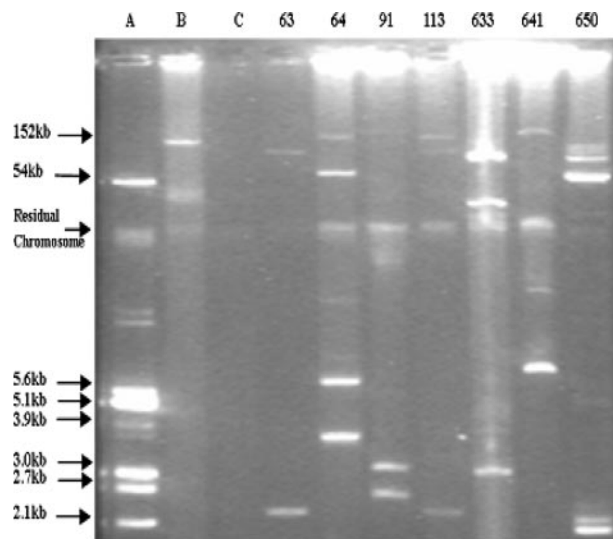

FIG. 1. Plasmid DNAs from clinical and reference strains. (A) *E. coli* V517. (B) *E. coli* J53 p*lac*. (C) *E. coli* J53. 63, 64, 91, 113, 633, 641, and 650 are clinical strains.

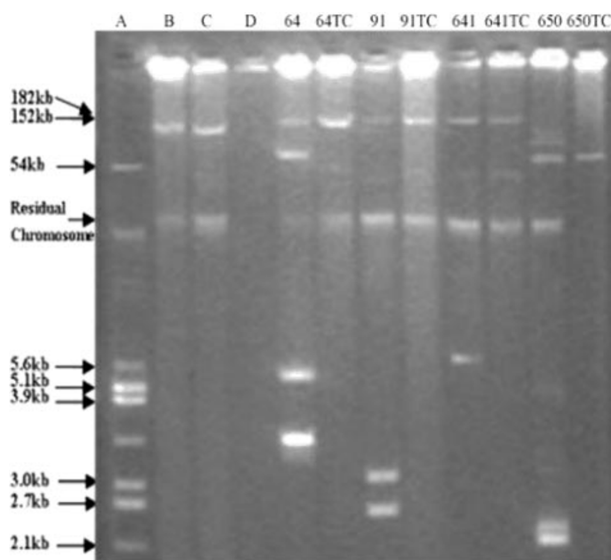

FIG. 2. Plasmid DNA of donors and transconjugants. (A) *E. coli* V517. (B) *E. coli* J53 p*lac*. (C) *E. coli* J53 R27. (D) *E. coli* J53. 64, 64TC, 91, 91TC, 641, 641TC, 650, and 650TC are clinical strains and transconjugants.

(GenBank accession numbers AY259085 and AY259086), which has been designated *qnrA1* (11).

Of seven *qnrA1*-positive clinical strains, four strains (strains 91, 113, 633, and 641) with cefotaxime MICs of 32 to ≥128 μg/ml carried ESBL genes (three strains with CTX-M-9 and one strain with CTX-M-14); strains 63 and 633 also carried the *ampC* β-lactamase genes ACT-1 and DHA-1, respectively. The other two strains with cefotaxime MICs of 1 to 2 μg/ml were negative for all β-lactamase genes tested. Transconjugants J53 pHS4 and J53 pHS5, with cefotaxime MICs of 8 μg/ml, were positive for CTX-M-9, and the other two transconjugants with cefotaxime MICs of ≤0.06 to 0.125 μg/ml were negative for the β-lactamase genes tested.

QRDR sequencing. The MICs of ciprofloxacin in *qnrA1*-bearing clinical strains were much higher than those of the corresponding transconjugants, with MICs of 4 to ≥128 μg/ml and 0.094 to 1 μg/ml, respectively. QRDRs in clinical strains and transconjugants were sequenced in order to determine if target modification occurs in *qnrA*-bearing clinical strains as it can in laboratory strains (10). Point mutations in *gyrA* were found with the resulting amino acid substitutions Ser83Ile, Ser83Leu, and Thr83Ile in *E. cloacae*, *E. coli*, and *C. freundii*, respectively, and Asp87Asn in *E. coli*. In *parC*, the substitution Ser80Ile was found in five strains. Five clinical strains with ciprofloxacin MICs of 32 to ≥128 μg/ml had two to three mutations in the *gyrA* and *parC* genes, and two strains of *E. coli* (strains 91 and 641) with ciprofloxacin MICs of 4 μg/ml exhibited no mutation in either gene.

Detection of *aac(6′)-Ib*. *aac(6′)-Ib* and *aac(6′)-Ib-cr* were evaluated to see if *aac(6′)-Ib-cr* caused the 11-fold difference in ciprofloxacin MICs among the transconjugants. The *aac(6′)-Ib-cr* gene, a variant of *aac(6′)-Ib* that confers reduced susceptibility to ciprofloxacin by N acetylation of its piperazinyl amine, was present in *E. coli* J53Az^R pHS3 and *E. coli* J53Az^R pHS6. *E. coli* J53Az^R pHS4 and *E. coli* J53Az^R pHS5 also contained an *aac(6′)-Ib* gene but not the *aac(6′)-Ib-cr* variant. The two J53 transconjugants with *aac(6′)-Ib-cr* had higher ciprofloxacin MICs (0.25 or 1.0 μg/ml) than the transconjugants with *aac(6′)-Ib* (MICs of 0.094 to 0.125 μg/ml), suggesting that *aac(6′)-Ib-cr* contributed to the decrease in ciprofloxacin susceptibility. The MICs of levofloxacin conferred by pHS3 were the same as those conferred by pHS4 and pHS5, indicating that the resistance against levofloxacin, which lacks an unsubstituted piperazinyl nitrogen, was unaffected by *aac(6′)-Ib-cr* (19) (Table 4).

***qnrA* RNA expression.** There remained, however, a fourfold difference in the MICs of ciprofloxacin (0.25 and 1.0 μg/ml, respectively) between transconjugants J53 pHS6 and J53 pHS3, which both harbored *qnrA1* and *aac(6′)-Ib-cr*. To determine whether the difference in ciprofloxacin MICs was caused by different levels of *qnrA1* transcripts, the level of *qnrA1*-specific transcripts was determined by quantitative RT-PCR. The relative expression levels of *qnrA1* in transconjugants were similar, from 1.0 to 2.5, but the relative expression level in J53 pHS6 was 32.5, much higher than the above-mentioned three transconjugants (Table 4). This result indicated that the higher ciprofloxacin MIC in transconjugant J53 pHS6 might be related to the higher *qnrA1* expression level than that in J53 pHS3.

Effect of an upstream DNA sequence on promoter strength. A fragment containing the promoter sequence of *qnrA1*-bearing plasmids was cloned upstream of the promoterless *cat* gene in reporter plasmid pKK232-8. The resulting plasmids, pKK232-8-HS3, pKK232-8-HS4, pKK232-8-HS5, pKK232-8-HS6, and pKK232-8, were transformed into *E. coli* HB101 competent cells. Chloramphenicol acetyltransferase activity is proportional to the strength of the promoter sequence. Chloramphenicol acetyltransferase determination was then esti-

TABLE 4. Characteristics of four *qnrA*-bearing plasmids in *E. coli* J53[a]

Transconjugant	Original clinical strain	Size of plasmid (kb)	MIC (μg/ml)												Presence of *aac(6′)-Ib-cr*	Relative expression level of *qnrA*
			CIP	LEV	AMP	CTX	CAZ	GEN	STR	KAN	AMK	TOB	SMZ	TMP		
E. coli J53 pHS3	*C. freundii* 64	>150	0.25	0.19	512	≤0.06	0.125	8	2	8	2	8	≥512	≤0.06	+	1.2
E. coli J53 pHS4	*E. cloacae* 91	>150	0.094	0.19	≥512	8	8	2	8	64	4	8	≥512	≥512	−	1.0
E. coli J53 pHS5	*E. cloacae* 641	>150	0.125	0.19	≥512	8	32	2	8	64	4	8	≥512	≥512	−	2.5
E. coli J53 pHS6	*E. coli* 650	~65	1.0	0.38	512	0.125	0.125	0.25	1	16	2	8	≥512	0.25	+	32.5
Recipient J53Az^R	NA	NA	0.008	0.012	8	≤0.06	0.125	0.25	1	0.5	0.5	0.25	8	≤0.06	NA	NA

[a] CIP, ciprofloxacin; AMP, ampicillin; CAZ, ceftazidime; CTX, cefotaxime; GEN, gentamicin; STR, streptomycin; KAN, kanamycin; AMK, amikacin; TOB, tobramycin; SMZ, sulfamethoxazole; TMP, trimethoprim; NA, not applicable.

```
                                                                          →Start qnrA
In36  TTCGGATGAGGAGCAAAAAGGTGGTTTATACTTCCTATACCCGTTAGCACCCTCCCTGATTAAAGGAAGCCGTATGGATATT
pHS3  TTCGGATGAGGAGCAAAAAGGTGGTTTATACTTCCTATACCCGTTAGCACCCTCCCTGATTAAAGGAAGCCGTATGGATATT
pHS4  TTCGGATGAGGAGCAAAAAGGTGGTTTATACTTCCTATACCCGTTAGCACCCTCCCTGATTAAAGGAAGCCGTATGGATATT
pHS5  TTCGGATGAGGAGCAAAAAGGTGGTTTATACTTCCTATACCCGTTAGCACCCTCCCTGATTAAAGGAAGCCGTATGGATATT
In37  TTCGGATGAGGAGCAAAAAGGTGGTTTATACTTCCTATACCC---------CCCTCCCTGATTAAAGGAAGCCGTATGGATATT
pHS6  TTCGGATGAGGAGCAAAAAGGTGGTTTATACTTCCTATACCC---------CCCTCCCTGATTAAAGGAAGCCGTATGGATATT
      -35                     -10        +1                                    M  D  I
```

FIG. 3. The *qnrA* promoter-like sequence of integrons (In*36* and In*37*) and plasmids pHS3, pHS4, pHS5, and pHS6. The deduced amino acid sequences are designed in single-letter code below the nucleotide. The sequences are similar to the promoter structure for *qnrA* expression in pQR1. The transcription orientation of the *qnrA* gene is indicated by the horizontal arrow. The −35 and −10 promoter sequences are boxed, as is the +1 transcription initiation site.

mated by measuring the chloramphenicol MIC. The chloramphenicol MICs for HB101 cells and transformant with pKK232-8 were both 3 μg/ml. In contrast, the MICs of chloramphenicol were 8 μg/ml for transformants with pKK232-8-HS3, pKK232-8-HS4, and pKK232-8-HS5 and 96 μg/ml for the transformant with pKK232-8-HS6, an MIC that was 12-fold higher than that of the above-mentioned other three transformants. This result indicated that the promoter in plasmid pHS6 (ciprofloxacin MIC of 1.0 μg/ml) was 12-fold stronger than that in plasmids pHS3 (ciprofloxacin MIC of 0.25 μg/ml) and pHS4 and pHS5 (ciprofloxacin MICs of 0.094 to 0.125 μg/ml).

Sequences of *qnrA1* promoters. The sequences of the cloned fragments upstream of *qnrA1* between it and ORF513 from different plasmids were sequenced (Fig. 3). The sequences upstream of *qnrA1* fragments from pHS3, pHS4, and pHS5 were identical to In*36* (GenBank accession number AY259085), and the sequence from pHS6 was identical to In*37* (accession number AY259086). Just like the difference of ciprofloxacin MICs between plasmids pHS6 and pHS3, the ciprofloxacin MIC for a strain carrying a plasmid carrying In*37* is fourfold higher than that of a strain carrying a plasmid with In*36* (MIC of 1 μg/ml versus 0.25 μg/ml) (22). The sequences differed by 7 bp (GTTAGCA) between the +1 transcription initiation site and the start of *qnrA1*, with these 7 bp absent in pHS6 and In*37* compared to pHS3 and In*36* (Fig. 3).

DISCUSSION

In this study, all consecutive gram-negative clinical strains that were resistant or intermediate to ciprofloxacin and that were isolated in a teaching hospital of Fudan University in Shanghai in 2005 were screened for *qnrA*, regardless of their susceptibilities to other antimicrobials. The results thus reflect the overall prevalence of *qnrA* in clinical isolates with reduced susceptibility to ciprofloxacin. *qnrA* continues to be present in consecutive clinical isolates of gram-negative bacteria in Shanghai. The prevalence now appears to be higher in *Enterobacter* spp. (31% 4/13) than in other species of clinical strains. *qnrA* was detected in 11% to 17% of strains of *Enterobacter* spp. isolated in the United States recently (17, 18). *qnrA* was positive in 15 of 47 (32%) blood culture isolates of *Enterobacteriaceae* resistant to both ciprofloxacin and cefotaxime collected in the United Kingdom. Of 15 *qnrA*-positive strains, 9 strains were *E. cloacae*, with a positive rate 56% (9/16) (3). An outbreak of *E. cloacae* infections occurred in a medical center in The Netherlands in 2002. All 83 outbreak strains were resistant to tobramycin and ceftriaxone, and 43% were both resistant and intermediate resistant to ciprofloxacin. *qnrA1* was present in 78 (94%) of these 83 isolates (12). In a recent report (23), *qnr* genes were positive in 16.3% (86/526) of clinical strains of *E. cloacae* isolated in Taiwan. *qnrA1*, *qnrB2*, and *qnrS1* genes were detected alone or in combination in 0.6%, 10.1%, and 6.5% of isolates, respectively. In this study, *qnrA1* was detected in 2% of 98 strains of *E. coli* and in 8% of those that were highly resistant to ciprofloxacin (MICs of 8 to ≥256 μg/ml) collected in the same hospital from 2000 to 2001 (22). *qnrA* was not found in a relatively large number of strains (232 strains) of nonfermenting bacilli including 128 strains of *P. aeruginosa* and 77 strains of *A. baumannii* in this population.

Most *qnrA*-bearing plasmids in *E. coli* J53 conferred an MIC of ciprofloxacin of 0.25 μg/ml. However, the *qnrA* plasmids from clinical *E. coli* isolates collected in Shanghai provided different levels of ciprofloxacin MICs, from 0.125 μg/ml to 2 μg/ml (22). In a study (19) of these plasmids, a variant of aminoglycoside acetyltransferase, *aac(6′)-Ib-cr*, that was able to N-acetylate ciprofloxacin and norfloxacin at the secondary amino nitrogen on their piperazinyl substituent was found. *aac(6′)-Ib-cr* causes low-level resistance to certain fluoroquinolones and acts in concert with *qnrA*. *aac(6′)-Ib-cr* (GenBank accession number AY259086) was first reported in 2003 in a *qnrA*-bearing plasmid, pHSH2, but it was found that *aac(6′)-Ib-cr* was widespread geographically and stable over time in the United States. Nine (11%) of the 78 *E. coli* strains from Shanghai carried non-*cr* variants of *aac(6′)-Ib*, and 40 (51%) harbored the *cr* variant allele of *aac(6′)-Ib* (16). Among 313 strains of *Enterobacteriaceae* collected from North America, 50% carried *aac(6′)-Ib*, and of these strains, 28% carried the *cr* variant (13). In the present study, *aac(6′)-Ib-cr* was detected in two (pHS3 and pHS6) of the four *qnrA*-bearing plasmids. The ciprofloxacin resistance level conferred by pHS3 and pHS6 was higher than the resistance conferred by the other two *cr* variant-negative plasmids, pHS4 and pHS5. This result supports previous reports that *aac(6′)-Ib-cr* and *qnrA* act additively to generate ciprofloxacin resistance (19). The levofloxacin resistance level was not affected by *aac(6′)-Ib-cr*.

qnrA RNA expression results indicated that the different ciprofloxacin MICs conferred by two *qnrA1*- and *aac(6′)-Ib-cr*-carrying plasmids, pHS3 and pHS6, were associated with the differential transcription of the *qnrA* gene. Differences in *qnrA*

transcription have also previously been claimed among *qnrA* plasmids (20).

Further study on the strength of the promoter indicated that different promoter strengths may cause the difference in ciprofloxacin MICs conferred by pHS6 and pHS3. Between the promoter and the start of *qnrA*, seven nucleotides, GTTA GCA, were missing in pHS6, a difference also found between In*37* and In*36*, which are similarly associated with differences in MICs of ciprofloxacin. This DNA difference may contribute to the difference in promoter strength between pHS6 and pHS3. Fournier et al. (4) previously reported that mutations or other DNA changes within or around the consensus sequences of promoters caused the difference in promoter strengths. Three different mutations in the consensus sequences of promoters resulted in a 4- to 31-fold increase in promoter strength compared to that of the wild-type promoter. The same study also reported that a change from 17 to 16 bp between the −35 and −10 consensus sequences resulted in a ninefold decrease in the promoter strength. A G→A mutation 3 bp downstream of the −10 consensus sequence seemed to increase the promoter strength by about twofold (4).

Mutations in the QRDR of *gyrA* and *parC* were detected in five of seven *qnrA*-bearing clinical strains with higher ciprofloxacin MICs. This finding is consistent with other reports. A previously reported study indicated that 25 of 28 (89%) clinical strains of *E. cloacae* and other enterobacterial species that harbored *qnrA* had mutations in the GyrA gyrase subunit and ParC topoisomerase subunit (8). A ciprofloxacin-resistant (MIC > 32 μg/ml) *E. coli* isolate, isolate 1B, was isolated from a urinary specimen of a Canadian patient treated with norfloxacin for infection due to a ciprofloxacin-susceptible isolate, isolate 1A. Both isolates harbored *qnrA1* and $bla_{\text{VEB-1}}$ genes. Isolate 1B had amino acid substitutions in gyrase and topoisomerase, already known to be responsible for resistance to quinolones (15).

In conclusion, *qnrA* continues to be present in clinical isolates of gram-negative bacilli in Shanghai, China. The prevalence of *qnrA* now appears to be higher in *E. cloacae* (31%) than in other species. *qnrA* has not yet been found in nonfermenting bacilli. The coexistence of both *qnrA* and *aac*(*6′*)*-Ib-cr* in a single plasmid and the differential transcription of the *qnrA* gene appear to contribute to differences in the levels of plasmid-encoded ciprofloxacin resistance.

ACKNOWLEDGMENTS

This work was supported by grant no. 2005CB0523101 (to M.W.) from the National Basic Research Program of China from the Ministry of Science and Technology, China, and grant no. 30440061 and 30572229 (to M.W.) from National Natural Science Foundation of China.

We thank George A. Jacoby and David C. Hooper for providing reference strains UAB1, *E. coli* J53Az^{R}, *E. coli* V517, *E. coli* R1, *E. coli* p*lac*, and *E. coli* R27 and for critical reviews of the manuscript.

REFERENCES

1. **Cambau, E., C. Lascols, W. Scougakoff, C. Bebear, R. Bonnet, J. D. Cavallo, L. Gutmann, M. C. Ploy, V. Jarlier, C. J. Soussy, and J. Jobert.** 2006. Occurrence of *qnrA*-positive clinical isolates in French teaching hospitals during 2002–2005. Clin. Microbiol. Infect. **12:**1013–1020.
2. **Clinical and Laboratory Standards Institute.** 2005. Performance standards for antimicrobial susceptibility testing, 15th informational supplement (M100-S15). Clinical and Laboratory Standards Institute, Wayne, PA.
3. **Corkill, J. E., J. J. Anson, and C. A. Hart.** 2005. High prevalence of the plasmid-mediated quinolone resistance determinant *qnrA* in multidrug-resistant *Enterobacteriaceae* from blood cultures in Liverpool, UK. J. Antimicrob. Chemother. **56:**1115–1117.
4. **Fournier, B., A. Gravel, D. C. Hooper, and P. H. Roy.** 1999. Strength and regulation of the different promoters for chromosomal β-lactamases of *Klebsiella oxytoca*. Antimicrob. Agents Chemother. **43:**850–855.
5. **Hata, M., M. Suzuki, M. Matsumoto, M. Takahashi, K. Sato, S. Ibe, and K. Sakae.** 2005. Cloning of a novel gene for quinolone resistance from a transferable plasmid in *Shigella flexneri* 2b. Antimicrob. Agents Chemother. **49:** 801–803.
6. **Jacoby, G. A.** 2005. Mechanisms of resistance to quinolones. Clin. Infect. Dis. **41**(Suppl. 2)**:**S120–S126.
7. **Jacoby, G. A., K. E. Walsh, D. M. Mills, V. J. Walker, Oh, H., A. Robicsek, and D. C. Hooper.** 2006. *qnrB*, another plasmid-mediated gene for quinolone resistance. Antimicrob. Agents Chemother. **50:**1178–1182.
8. **Lascols, C., J. Robert, V. Cattoir, C. Bebear, J. D. Cavallo, I. Podglajen, M. C. Ploy, R. Bonnet, C. J. Soussy, and E. Cambau.** 2007. Type II topoisomerase mutations in clinical isolates of *Enterobacter cloacae* and other enterobacterial species harbouring the *qnrA* gene. Int. J. Antimicrob. Agents **29:**402–409.
9. **Mammeri, H., M. V. De Loo, L. Poirel, L. Martinez-Martinez, and P. Nordmann.** 2005. Emergence of plasmid-mediated quinolone resistance in *Escherichia coli* in Europe. Antimicrob. Agents Chemother. **49:**71–76.
10. **Martinez-Martinez, L., A. Pascual, and G. A. Jacoby.** 1998. Quinolone resistance from a transferable plasmid. Lancet **351:**797–799.
11. **Nordmann, P., and L. Poirel.** 2005. Emergence of plasmid-mediated resistance to quinolones in *Enterobacteriaceae*. J. Antimicrob. Chemother. **56:** 463–469.
12. **Paauw, A., A. C. Fluit, J. Verhoef, and M. A. Leverstein-van Hall.** 2006. *Enterobacter cloacae* outbreak and emergence of quinolone resistance gene in Dutch hospital. Emerg. Infect. Dis. **12:**807–812.
13. **Park, C. H., A. Robicsek, G. A. Jacoby, D. Sahm, and D. C. Hooper.** 2006. Prevalence in the United States of aac*(6′)-Ib-cr* encoding a ciprofloxacin-modifying enzyme. Antimicrob. Agents Chemother. **50:**3953–3955.
14. **Perez-Perez, F. J., and N. D. Hanson.** 2002. Detection of plasmid-mediated AmpC β-lactamase genes in clinical isolates by using multiplex PCR. J. Clin. Microbial. **40:**2153–2162.
15. **Poirel, L., J. D. Pitout, L. Calvo, J. M. Rodriguez-Martinez, D. Church, and P. Nordmann.** 2006. In vivo selection of fluoroquinolone-resistant *Escherichia coli* isolates expressing plasmid-mediated quinolone resistance and expanded-spectrum β-lactamase. Antimicrob. Agents Chemother. **50:**1525–1527.
16. **Robicsek, A., G. A. Jacoby, and D. C. Hooper.** 2006. The worldwide emergence of plasmid-mediated quinolone resistance. Lancet Infect. Dis. **6:**629–640.
17. **Robicsek, A., D. F. Sahm, J. Strahilevitz, G. A. Jacoby, and D. C. Hooper.** 2005. Broader distribution of plasmid-mediated quinolone resistance in the United States. Antimicrob. Agents Chemother. **49:**3001–3003.
18. **Robicsek, A., J. Strahilevitz, D. F. Sahm, G. A. Jacoby, and D. C. Hooper.** 2006. *qnr* prevalence in ceftazidime-resistant *Enterobacteriaceae* isolates from the United States. Antimicrob. Agents Chemother. **50:**2872–2874.
19. **Robicsek, A., J. Strahilevitz, G. A. Jacoby, M. Macielag, D. Abbanat, C. H. Park, K. Bush, and D. C. Hooper.** 2006. Fluoroquinolone-modifying enzyme: a new adaptation of a common aminoglycoside acetyltransferase. Nat. Med. **12:**83–88.
20. **Rodríguez-Martínez, J. M., C. Velasco, A. Pascual, I. Garcia, and L. Martinez-Martinez.** 2006. Correlation of quinolone resistance levels and differences in basal and quinolone-induced expression from three *qnrA*-containing plasmids. Clin. Microbiol. Infect. **12:**440–445.
21. **Wang, M., D. F. Sahm, G. A. Jacoby, and D. C. Hooper.** 2004. Emerging plasmid-mediated quinolone resistance associated with the *qnr* gene in *Klebsiella pneumoniae* clinical isolates in the United States. Antimicrob. Agents Chemother. **48:**1295–1299.
22. **Wang, M., J. H. Tran, G. A. Jacoby, Y. Y. Zhang, F. Wang, and D. C. Hooper.** 2003. Plasmid-mediated quinolone resistance in clinical isolates of *Escherichia coli* from Shanghai, China. Antimicrob. Agents Chemother. **47:**2242–2248.
23. **Wu, J. J., W. C. Ko, S. H. Tsai, and J. J. Yan.** 2007. Prevalence of plasmid-mediated quinolone resistance determinants QnrA, QnrB, and QnrS among clinical isolates of *Enterobacter cloacae* in a Taiwanese hospital. Antimicrob. Agents Chemother. **51:**1223–1227.