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qnrB, Another Plasmid-Mediated Gene for Quinolone Resistance

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A novel plasmid-mediated quinolone resistance gene, *qnrB*, has been discovered in a plasmid encoding the CTX-M-15 β-lactamase from a *Klebsiella pneumoniae* strain isolated in South India. It has less than 40% amino acid identity with the original *qnr* (now *qnrA*) gene or with the recently described *qnrS* but, like them, codes for a protein belonging to the pentapeptide repeat family. Strains with *qnrB* demonstrated low-level resistance to all quinolones tested. The gene has been cloned in an expression vector attaching a polyhistidine tag, which facilitated purification to ≥95% homogeneity. As little as 5 pM of QnrB-His$_6$ protected purified DNA gyrase against inhibition by 2 μg/ml (6 μM) ciprofloxacin. With a PCR assay *qnrB* has been detected in *Citrobacter koseri*, *Enterobacter cloacae*, and *Escherichia coli* isolates from the United States, linked to SHV-12 β-lactamase and coding for a product differing in five amino acids from the Indian (now QnrB1) variety. The *qnrB* gene has been found near Orf1005 in some, but not all, plasmids and in association with open reading frames matching known chromosomal genes, suggesting that it too was acquired by plasmids from an as-yet-unknown bacterial source.

The first plasmid-mediated quinolone resistance gene (*qnr*) was discovered in a *Klebsiella pneumoniae* isolate from Birmingham, Alabama, collected in 1994 (10). It occurred in a multiresistance plasmid, pMG252, in an integron-like structure near Orf513 (17). Qnr, the gene product, is a member of the pentapeptide repeat family of proteins and has been shown to block the action of ciprofloxacin on purified DNA gyrase and topoisomerase IV (17, 19). In *Escherichia coli* pMG252 determines low-level quinolone resistance but facilitates the selection of higher-level resistance mutations (10). *qnr* plasmids have been found in clinical isolates of *Citrobacter freundii*, *Enterobacter* spp., *E. coli*, *K. pneumoniae*, *Providencia stuartii*, and *Salmonella* spp. from the United States, Europe, and the Near and Far East (1, 13). Another *qnr* gene, *qnrS*, has also recently been found in a plasmid from a strain of *Shigella flexneri* isolated in Japan (2).

While investigating strains of *K. pneumoniae* from India, some of which contained *qnr*, it was realized that several could transfer low-level quinolone resistance but were negative by PCR for *qnr*. The new plasmid-mediated quinolone resistance gene has been termed *qnrB*, and the original gene is now designated *qnrA*. *qnrB* has been cloned and sequenced. Purified QnrB protects DNA gyrase from quinolone action like QnrA does. A PCR assay for *qnrB* indicates that it is as common as *qnrA* in samples from the United States and has greater amino acid variability.

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MATERIALS AND METHODS

Strains, plasmids, and growth conditions. *K. pneumoniae* strains 17, 19, 21, 24, and 25 were isolated from blood, a catheter tip, pus, and sputum in 2002 and 2003 at Coimbatore in South India. *Citrobacter koseri* 3699-1 was isolated at Long Beach, CA, in 2000. *Enterobacter cloacae* 3668 came from Philadelphia, PA, in 2000-2001 (9). *E. cloacae* 78149 and *Escherichia coli* EC-35 were acquired in 1996 from St. Louis, MO. Matings were performed with *E. coli* J53 Azi^r (*met pro*; azide-resistant) as a recipient (6). *E. coli* TOP10 (Invitrogen, Carlsbad, CA) was used in cloning. Strains were routinely grown in Luria-Bertani broth. Culture plates contained tryptic soy agar (TSA) or Mueller-Hinton agar (Becton, Dickinson and Co., Sparks, MD). Selective media contained ampicillin (100 μg/ml), cefotaxime (10 μg/ml), chloramphenicol (25 μg/ml), ciprofloxacin (0.015 μg/ml), kanamycin (25 μg/ml), or nalidixic acid (12 μg/ml) as required.

Susceptibility testing. Disk and agar dilution susceptibility testing was performed as described in CLSI (formerly NCCLS) publications, using Mueller-Hinton agar and 16 to 20 h of incubation at 37°C (11, 12).

Cloning and nucleotide sequence analysis. Plasmid DNA was isolated from an *E. coli* J53 derivative by using the Large-Construct kit (QIAGEN, Valencia, CA), digested with one of several endonucleases, ligated to similarly restricted phagemid pBC SK (Stratagene), and introduced into *E. coli* TOP10 with selection on TSA plates containing chloramphenicol and nalidixic acid. Using the GPS-1 genome priming system (NE Biolabs, Ipswich, MA), a kanamycin resistance transposon was inserted to inactivate the quinolone resistance gene and allow sequencing using primerN and primerS from the transposon ends. Cycle sequencing was carried out with an ABI Prism 3100 genetic analyzer (Tufts University Core Facility) and was continued by primer walking on both DNA strands. For sequence comparisons, the NCBI BLAST program and facilities of the TIGR Comprehensive Microbial Resource (www.tigr.org) were utilized.

Overexpression and purification of QnrB. The *qnrB1* gene from pMG298 was cloned into expression vector pQE-60 (QIAGEN) at its NcoI and BamHI sites. An internal NcoI site was first removed from *qnrB1* by using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) with primers 5′-GTGATTTATCGATGGCGGATTTTCGC and 5′-GCGAAAATCCGCCATCGATAAATCAC, which cause no change in the amino acid sequence. The gene was then amplified by PCR using primers 5′-GGCCATGGCGCCATTACTGTATAAA and 5′-GCGCGGATCCACCAATCACCGCGATGCC and ligated after digestion with NcoI and BamHI into pQE-60. In the process, the amino acid following the initial methionine was changed from threonine to alanine and a tag of six histidines was added to the C terminus of the protein. Proper construction was confirmed by sequencing, and the pQE60-QnrB1 plasmid was transformed into *E. coli* M15(pREP4) (QIAGEN) to place expression of *qnrB1* under the control of induction by isopropyl-1-thio-β-D-galactopyranoside (IPTG).

E. coli M15 (pREP4)(pQE-60-QnrB1) was grown in LB medium with 100 μg/ml ampicillin and 25 μg/ml kanamycin until the cell density reached an optical density at 600 nm of 0.6 to 0.8, induced with IPTG at a final concentration of 1 mM, and allowed 4 h of further growth until being harvested by centrifugation. The pellet was suspended in 20 mM Tris-HCl (pH 7.5), 10% glycerol, 150 mM NaCl and lysed with 0.02% lysozyme and 1× EDTA-free protease inhibitor mix (Roche Diagnostics, Mannheim, Germany) on ice for 0.5 to 2 h. The lysate was centrifuged at 25,000 × *g* for 90 min at 4°C. The supernatant containing the soluble

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      1                                                  50
 QnrA ---------MDIIDKVFQQEDFSRQDLSDSRFRRCRFYQCDFSHCQLQDA
QnrB1 MTPLLYKKTGTNMALALVGEKIDRNRFTGEKIENSTFFNCDFSGADLSGT
QnrB2 ------------MALALVGEKINRNRFTGEKIENSTFFNCDFSGADLSGT
 QnrS ---------METYNHTYRHHNFSHKDLSDLTFTACTFIRSDFRRANLRDT
      51                                                 100
 QnrA SFEDCSFIESGAVEGCHFSYADLRDASFKACRLSLANFSGANCFGIEFRE
QnrB1 EFIGCQFYDRESQKGCNFSRAMLKDAIFKSCDLSMADFRNSSALGIEIRH
QnrB2 EFIGCQFYDRESQKGCNFSRAMLKDAIFKSCDLSMADFRNASALGIEIRH
 QnrS TFVNCKFIEQGDIEGCHFDVADLRDASFQQCQLAMANFSNANCYGIEFRA
      101                                                150
 QnrA CDLKGANFSRARFYNQVSHKMYFCSAYISGCNLAYTNLSGQCLEKCELFE
QnrB1 CRAQGADFRGASFMNMITTRTWFCSAYITNTNLSYANFSKVVLEKCELWE
QnrB2 CRAQGADFRGASFMNMITTRTWFCSAYITNTNLSYANFSKVVLEKCELWE
 QnrS CDLKGANFSRTNFAHQVSNRMYFCSAFISGCNLSYANMERVCLEKCELFE
      151                                                200
 QnrA NNWSNANLSGASLMGSDLSRGTFSRDCWQQVNLRGCDLTFADLDGLDPRR
QnrB1 NRWIGAQVLGATFSGSDLSGGEFSTFDWRAANFTHCDLTNSELGDLDIRG
QnrB2 NRWMGAQVLGATFSGSDLSGGEFSTFDWRAANFTHCDLTNSELGDLDIRR
 QnrS NRWIGTNLAGASLKESDLSRGVFSEDVWGQFSLQGANLCHAELDGLDPRK
      201                  228
 QnrA VNLEGVKICAWQQEQLLEPLGVIVLPD-
QnrB1 VDLQGVKLDNYQASLLMERLGIAVIG--
QnrB2 VDLQGVKLDNYQASLLMERLGIAIIG--
 QnrS VDTSGIKIAAWQQELILEALGIVVYPD-
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FIG. 1. CLUSTAL W alignment of QnrA, QnrB1, QnrB2, and QnrS.

protein was filtered through a 0.2-μm membrane with a Nalgene filter unit (Nalgene, Rochester, NY) and loaded onto a preequilibrated HiTrapChelating HP column (Amersham Biosciences, Piscataway, NJ), and the histidine-tagged protein was eluted with increasing concentrations of imidazole (50 to 300 mM) and collected in 1-ml fractions. The eluted fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using precast 12% Ready Tris-HCl gels (Bio-Rad, Hercules, CA). Fractions containing a single protein band of 25 kDa were dialyzed against 50 mM Tris-HCl (pH 7.5), 5 mM dithiothreitol, 150 mM NaCl, 10% glycerol overnight at 4°C with several buffer changes. Protein concentrations were determined using the Bradford assay (Bio-Rad).

Gyrase supercoiling assay. DNA supercoiling assays were performed as described previously, using DNA gyrase reconstituted from cloned *E. coli* GyrA and GyrB subunits (18) and relaxed pBR322 plasmid DNA (Topogen Inc., Port Orange, FL).

β-Lactamase characterization. The presence of an extended-spectrum β-lactamase (ESBL) was suspected from cefotaxime and ceftazidime resistance and confirmed by repeat testing in the presence of clavulanic acid (11). Transmissibility of resistance was tested by mating clinical isolates with *E. coli* J53 Azi^r with selection on TSA agar plates containing 10 μg/ml cefotaxime and 200 μg/ml sodium azide. β-Lactamase isoelectric focusing was carried out using the Phast-System (Amersham Biosciences, Piscataway, NJ) as described by Huovinen (4). *bla* genes were amplified using primers 5′-TTTCCCCATTCCGTTTCCGC and 5′-TTCGTATCTTCCAGAATAAG for $bla_{\text{CTX-M-15}}$ amplification and previously described primers S1 and S2 for bla_{SHV} amplification (20). The amplified genes were sequenced using the same primers and additional primers designed from the known *bla* gene sequence.

PCR conditions. Detection of *qnrA* utilized primers QP1 and QP2 with PCR conditions as previously described (5). For *qnrB*, primers FQ1 (5′-ATGACGC CATTACTGTATAA) and FQ2 (5′-GATCGCAATGTGTGAAGTTT) were used. PCR conditions were 94°C for 45 s, 53°C for 45 s, and 72°C for 1 min for 32 cycles. The presence of Orf513 was tested with primers 5′-AAGGAACGCC ACGGCGAGTCAA and 5′-TGCAAAGACGCCGTGGAAGC, and the presence of Orf1005 was evaluated with primers 5′-CTTGGTATTCGAAGCT GGTC and 5′-CTACCGTTTGCAACAGTAAG.

Nucleotide sequence accession numbers. The *qnrB1* and *qnrB2* sequences have been submitted to GenBank with accession numbers DQ351241 and DQ351242.

RESULTS

Discovery of *qnrB*. The *K. pneumoniae* strains from India were originally studied to determine the basis of their ESBL phenotype. Transconjugants of strains 17, 19, 24, and 25, selected for cefotaxime resistance, were noted to have low-level quinolone resistance, but when tested by PCR with primers QP1 and QP2, only *K. pneumoniae* strains 19 and 24 were positive for the known *qnr* gene.

Accordingly, DNA of plasmid pMG298 from strain 17 was digested with PstI endonuclease and ligated into vector pBC SK (determining chloramphenicol resistance), selecting for simultaneous resistance to ciprofloxacin and chloramphenicol. A recombinant plasmid containing a 15.3-kb insert was obtained. Quinolone resistance was also expressed from pBC SK containing a 4.8-kb BamHI fragment from plasmid pMG299 originating in strain 24 and from a 5.9-kb PstI fragment from plasmid pMG300 transferred from strain 25.

To facilitate DNA sequencing, a Tn*7*-based transposon carrying a kanamycin resistance gene was inserted into the recombinant plasmids, and colonies were screened for loss of nalidixic acid resistance. Using primers that matched sequence at the ends of the inserted transposon, sequencing was initiated and continued by primer walking with the original unmodified recombinant plasmid. A new quinolone resistance gene, *qnrB1*, was discovered. It had 49.5% nucleotide identity and 39.5% amino acid identity with *qnrA* and 49.3% nucleotide identity and 37.4% amino acid identity with *qnrS* (Fig. 1) and had the potential to code for a 226-amino-acid protein, which belonged to the pentapeptide repeat family.

Distribution of *qnrB*. Using PCR with primers derived from the *qnrB1* sequence, the gene was found also in *K. pneumoniae* strains 21, 24, and 25 from southern India, but the plasmids involved differed in resistance properties (Table 1). Plasmid pMG299 from strain 24, like plasmid pMG298 from strain 17, carried $bla_{\text{CTX-M-15}}$, but the *qnrB1* plasmid pMG300 from strain 25 encoded SHV-12 and had no CTX-M gene by PCR. The $bla_{\text{SHV-12}}$ gene was also present in strain 21, but neither β-

TABLE 1. Properties of QnrB plasmids

Plasmid	Species or strain found in:	Location	β-Lactamase(s)	Other resistances[a]	Orf1005
pMG298	*K. pneumoniae* 17	Southern India	CTX-M-15	Cm Gm Km Sm Su Tc Tm Tp	+
	K. pneumoniae 21	Southern India	SHV-1 and SHV-12	Tra$^-$	−
pMG299	*K. pneumoniae* 24	Southern India	CTX-M-15	Ak Gm Km Sm Su Tm Tp	+
pMG300	*K. pneumoniae* 25	Southern India	SHV-12	Gm Km Sm Tm	+
pMG301	*C. koseri*	Long Beach, CA	SHV-12	Gm Sm Su Tp	−
pMG302	*E. cloacae*	Philadelphia, PA	SHV-12	Cm Gm Km Sm Su Tm Tp	−
pMG303	*E. cloacae*	St. Louis, MO	SHV-12	Cm Km Sm Su Tc Tp	−
pMG304	*E. coli*	St. Louis, MO	SHV-12	Cm Km Sm Su	−

[a] Resistance abbreviations: Ak, amikacin; Cm, chloramphenicol; Gm, gentamicin; Km, kanamycin; Sm, streptomycin; Su, sulfonamide; Tc, tetracycline; Tm, tobramycin; Tp, trimethoprim.

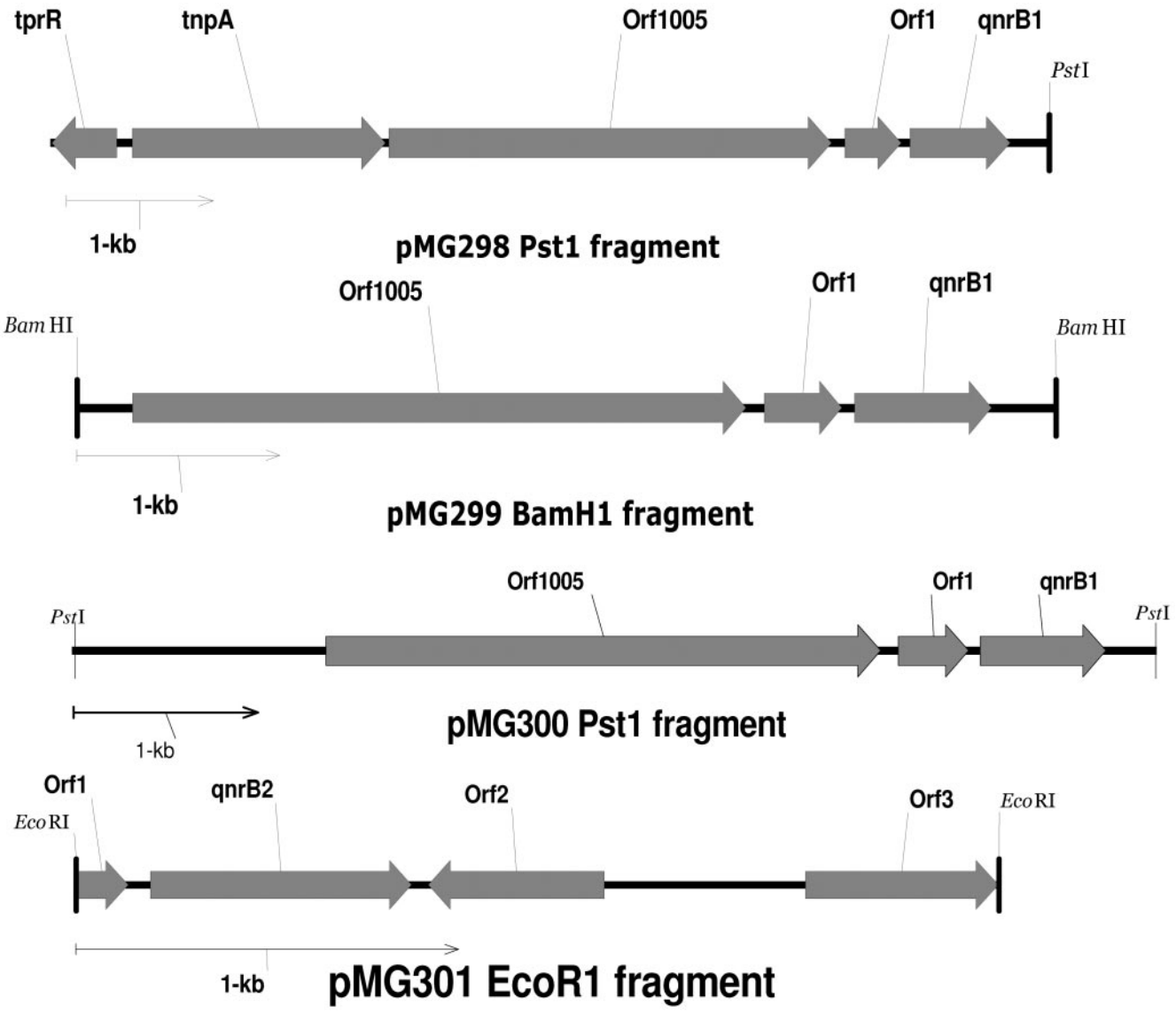

FIG. 2. Maps of cloned *qnrB* genes. The sizes of the cloned fragments vary, as indicated by the 1-kb reference bars. Orf1 resembles *pspF*, Orf2 is related to genes of unknown function in various gram-negative bacteria, and Orf3 resembles *sapA*.

lactam nor other antibiotic resistances could be transferred from it by conjugation, implying the presence of a Tra$^-$ plasmid.

Over 100 other plasmid-carrying strains were screened for *qnrB*. A strain with another CTX-M-15 plasmid was negative, but 4 of about 20 independently derived plasmids encoding SHV-12 were positive, including plasmids found in *C. koseri*, *E. cloacae*, and *E. coli* (Table 1). All had identical sequences which differed from that of *qnrB1* by 26 of 680 nucleotides, including an additional nucleotide prior to a second potential ATG start codon at position 36. The extra nucleotide would cause a frameshift if translation began at the first site, so that the *qnrB2* gene codes for a 215-amino-acid protein that in addition differs from QnrB1 in 5 amino acids (Fig. 1). The *qnrB2* plasmids all encode SHV-12 but differ in other associated resistances (Table 1). Attempts to clone *qnrB2* with BamHI or PstI failed, but the gene was cloned into pBC SK as a 2.4-kb EcoRI fragment.

Genetic environment of *qnrB*. The immediate genetic environments of *qnrB1* in plasmids pMG298, pMG299, and pMG300 were similar (Fig. 2). *qnrB1* was found downstream from Orf1005, which encodes a putative transposase and is brack-

TABLE 2. Susceptibility of *E. coli* J53 transconjugants to various quinolones

Plasmid in *E. coli* J53	Qnr protein	MIC (μg/ml)				
		Nalidixic acid	Ciprofloxacin	Gatifloxacin	Levofloxacin	Moxifloxacin
R$^-$		4	0.015	0.03	0.03	0.06
pMG252	QnrA	32	0.5	0.5	1	1
pHSH4-3	QnrA	16	0.25	0.5	0.5	0.5
pMG298	QnrB1	16	1	1	0.5	2
pMG299	QnrB1	16	0.25	0.5	0.5	1
pMG300	QnrB1	16	0.25	0.5	0.25	1

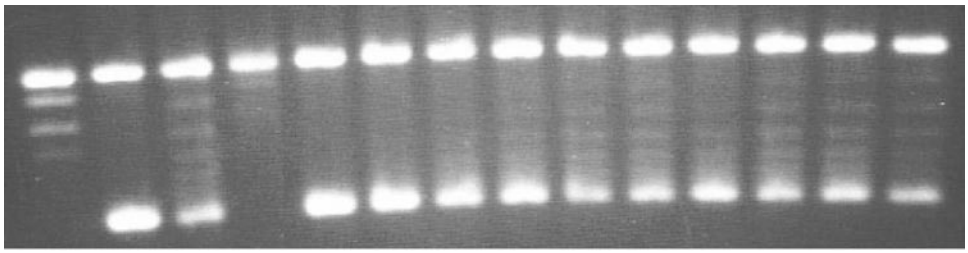

FIG. 3. QnrB1 protection of DNA gyrase from ciprofloxacin inhibition of supercoiling. Reaction mixtures of 30 μl were analyzed by agarose gel electrophoresis. Reaction mixtures contained 0.2 μg relaxed pBR322 DNA (lanes 1 to 14), 6.7 nM gyrase (lanes 2 to 14), 2 μg/ml ciprofloxacin (lanes 3 to 14), and QnrB-His_6 fusion protein at 25 μM (lane 4), 5 μM (lane 5), 2.5 μM (lane 6), 0.5 μM (lane 7), 50 nM (lane 8), 5 nM (lane 9), 0.5 nM (lane 10), 50 pM (lane 11), 5 pM (lane 12), or 0.5 pM (lane 13).

eted by imperfect 83-bp inverted repeat segments (16). In pMG298 a *tnpA* gene was found downstream from Orf1005, but this gene was absent in pMG300. Between Orf1005 and *qnrB1* was a 383-bp open reading frame, Orf1, which is >70% identical to a truncated *pspF* gene coding for the transcriptional activator of the stress-inducible *psp* operon (7). Orf1 contains an EcoRI site, and a shorter segment of Orf1 was found upstream from the *qnrB2* gene, which was cloned on an EcoRI fragment (Fig. 2). Downstream from *qnrB2* was an open reading frame (Orf2), with >60% identity to hypothetical proteins of several gram-negative species, and Orf3, with 83% identity to the *sapA* gene, which encodes a peptide transport periplasmic protein in gram-negative bacteria. By a PCR assay Orf1005 was present in *K. pneumoniae* strains 17, 24, and 25 but not in any of the strains containing *qnrB2*. Orf512 was not detected in any of the strains.

Effect of QnrB on quinolone susceptibility. Like QnrA, QnrB provided low-level resistance to all quinolones tested (Table 2).

Effect of QnrB on quinolone inhibition of gyrase. To investigate the mechanism of quinolone protection, *qnrB* was cloned into an expression vector that attached a C-terminal polyhistidine tag, which facilitated purification of QnrB-His_6 protein by Ni affinity chromatography. His-tagged QnrB appeared to be ≥95% homogenous by gel assay.

QnrB-His_6 demonstrated a concentration-dependent protection of purified gyrase from ciprofloxacin inhibition of DNA supercoiling (Fig. 3). With 2 μg/ml (6 μM) ciprofloxacin, the concentration of QnrB-His_6 required for half protection was about 0.5 nM, and a protective effect was seen with as little as 5 pM. The highest concentration of QnrB-His_6 tested (25 μM) inhibited gyrase-mediated DNA supercoiling, but inhibition was not seen with 5 μM QnrB-His_6, a concentration still 750 times higher than that of DNA gyrase (Fig. 3, compare lanes 4 and 5).

DISCUSSION

QnrB, like QnrA and QnrS (2), provides low-level resistance to quinolones and belongs to the pentapeptide repeat family of proteins, one member of which has recently been shown to have a DNA-like structure which would allow it to mimic DNA as a substrate for DNA gyrase (3). For a protein in which overall structure is important rather than catalytic activity, considerable amino acid variability may be permissible. Thus, QnrB and QnrA have only 39.5% of their amino acids in common, while QnrB and QnrS share 37.4%, but all are pentapeptide repeat proteins with two domains joined by a glycine residue (17).

Purified QnrB, like QnrA, protected DNA gyrase from quinolone action. It seems to be even more potent than QnrA in blocking the action of ciprofloxacin (17). At a concentration almost 4,000 times that of DNA gyrase, QnrB inhibited the enzyme, but this effect disappeared at a fivefold lower concentration of QnrB. Thus, in contrast to MfpA, another pentapeptide repeat protein that blocks ciprofloxacin action (3), QnrB did not inhibit gyrase-mediated DNA supercoiling over a wide range of quinolone-protective concentrations. Accordingly, models of Qnr action that do not require direct gyrase inhibition must be considered.

A close relative and likely progenitor of QnrA, differing in only 1 to 2% amino acids, has recently been found in the commensal water organism *Shewanella algae* (15). The origin of QnrB is not yet known, since the closest relative currently disclosed by a BLAST search is a hypothetical protein from *Photobacterium profundum* with only 44.5% amino acid identity. The *qnrA* gene has been found in plasmids with a variety of other resistance determinants but always as part of a *sul1*-type integron (13). *qnrB1* is located near a putative transposase, Orf1005, in plasmids pMG298 and pMG299, but Orf1005 was absent from QnrB2 plasmids from the United States. The linkage of *qnrB* to Orf1, Orf2, and Orf3, which resemble known chromosomal genes, suggests that all were acquired from a chromosomal source. The homologues of Orf1 and Orf3 are adjacent to each other on the *P. profundum* chromosome, but the homologues of *qnrB* and Orf2 occupy separate and distant locations. The mechanism of *qnrB* acquisition, like that of *qnrS*, remains to be elucidated.

In both India and the United States *qnrB* has been found on plasmids also encoding ESBLs: CTX-M-15 and SHV-12 in India and SHV-12 in the United States. The frequent association of quinolone resistance with ESBL production has been noted in several studies (8, 14). The presence of *qnr* and bla_{ESBL} genes on the same plasmid is one of several possible explanations for this association.

qnrB2 has been found on plasmids collected in 1996, and the

association of *qnrB* with SHV-12 on plasmids found in both India and the United States suggests that this resistance mechanism has been present long enough to disseminate widely. Preliminary surveys indicate that in ceftazidime-resistant *Enterobacter* and *Klebsiella* isolates from the United States *qnrB* is as common as *qnrA* (Robicsek et al., unpublished observations). It would not be surprising if even more members of the *qnr* family were discovered.

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