

# Transferable Resistance to Aminoglycosides by Methylation of G1405 in 16S rRNA and to Hydrophilic Fluoroquinolones by QepA-Mediated Efflux in *Escherichia coli*<sup>∇</sup>

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**Plasmid pIP1206 was detected in *Escherichia coli* strain 1540 during the screening of clinical isolates of *Enterobacteriaceae* for high-level resistance to aminoglycosides. The sequence of this IncFI conjugative plasmid of ca. 100 kb was partially determined. pIP1206 carried the *rmtB* gene for a ribosome methyltransferase that was shown to modify the N7 position of nucleotide G1405, located in the A site of 16S rRNA. It also contained the *qepA* (quinolone efflux pump) gene that encodes a 14-transmembrane-segment putative efflux pump belonging to the major facilitator superfamily of proton-dependent transporters. Disruption of membrane proton potential by the efflux pump inhibitor carbonyl cyanide *m*-chlorophenylhydrazone in a transconjugant harboring the *qepA* gene resulted in elevation of norfloxacin accumulation. The transporter conferred resistance to the hydrophilic quinolones norfloxacin and ciprofloxacin.**

Despite the development of new  $\beta$ -lactams and fluoroquinolones, aminoglycosides are still extensively used for the treatment of severe infections due to gram-negative bacteria.

Aminoglycosides act by causing translational errors and by inhibiting translocation (4). Their target sites include ribosomal domains in which the accuracy of the codon-anticodon is assessed (20), and in particular, they bind to a highly conserved motif of 16S rRNA (14, 28). Since their introduction into clinical use, five mechanisms of resistance to these drugs have been reported in bacterial human pathogens (12): (i) decreased intracellular accumulation of the antibiotic by alteration of outer membrane permeability, diminished inner membrane transport, or active efflux; (ii) enzymatic modification of the drug, which is the most common; (iii) modification of the target by mutation in ribosomal proteins or in 16S rRNA; (iv) trapping of the drug; and most recently, (v) posttranscriptional methylation of rRNA, using *S*-adenosyl-methionine as a cofactor (7). The last mechanism confers high-level resistance to all available aminoglycosides used for systemic therapy, except streptomycin. The first gene for this type of resistance, *armA*, was identified in France located on a self-transferable plasmid (7) and was shown to be part of the composite transposon Tn1548 (8) and to encode an enzyme that methylates the N7 position of nucleotide G1405 in 16S rRNA (10). Reports followed of four methyltransferases: RmtA (27) and RmtB (5), which share 82% identity; RmtC (24), with less than 29% identity with RmtA and RmtB; and, more recently, RmtD (6), sharing 40% and 42% identity with RmtA and RmtB, respectively, but less than 29% identity with RmtC. These methyltransferases are only 29% to 31% identical with ArmA.

Fluoroquinolones, by binding to complexes that form between DNA and type II topoisomerases, gyrase and topoisomerase IV, alter chromosome topology that is essential in replication, transcription, recombination, and DNA repair (9). Four mechanisms of resistance to quinolones have been described. The most common is mutational alteration in the so-called quinolone resistance-determining regions of the drug targets (9). The second is the reduction of fluoroquinolone accumulation by active export of the drugs via chromosomal (18) and, most recently, plasmid-borne (25) efflux pumps. Also recently, two additional plasmid-mediated low-level resistance mechanisms have been reported; the Qnr proteins, which protect type II topoisomerases from quinolones (13, 21), and AAC(6')-Ib-cr, a variant aminoglycoside acetyltransferase that modifies ciprofloxacin (22).

During the screening of clinical isolates of enterobacteria for high-level resistance to aminoglycosides, *Escherichia coli* 1540 was found to harbor the IncFI self-transferable plasmid pIP1206 of ca. 100 kb, which was partially sequenced. The plasmid carried the *rmtB* gene (2), and the methylation position of RmtB on 16S rRNA was determined. pIP1206 was also found to confer resistance to hydrophilic quinolones due to the presence of the quinolone efflux pump (*qepA*) gene, which encodes a putative drug efflux pump belonging to the major facilitator superfamily (MFS).

## MATERIALS AND METHODS

**Strains, plasmids, and growth conditions.** *E. coli* strain 1540 was isolated in a Belgian hospital during screening of 15,386 nonreplicative clinical isolates of *Enterobacteriaceae* for aminoglycoside resistance (2). *E. coli* C600Rif and TOP10 (Invitrogen, Paisley, United Kingdom) strains were used as recipients for conjugation and transformation, respectively. *E. coli* AG100A, inactivated in the AcrAB drug efflux pump (16), was used to determine antibiotic susceptibility. Plasmids pCR2.1, pCR-Blunt, and pBAD/His (Invitrogen) were used for cloning of PCR products. The strains were grown in brain heart infusion broth or on agar at 37°C.

**Susceptibility testing.** Antibiotic susceptibility was tested by disk diffusion on Mueller-Hinton agar, according to the Comité de l'Antibiogramme de la Société Française de Microbiologie standards (3). MICs of antimicrobial agents and dyes

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TABLE 1. MICs of antimicrobial agents and dyes for the strains

<i>E. coli</i> strain/plasmid	Efflux pump inhibitor <sup>b</sup>	MIC (μg/ml) <sup>a</sup>							
		NOR	CIP	PFX	GAT	LVX	MXF	NAL	EBR
C600Rif	0	0.064	0.023	0.094	0.023	0.032	0.064	2	128
	+NMP	0.047	0.016	0.064	0.016	0.016	0.032	1.5	64
	+PAβN	0.032	0.012	0.047	0.016	0.012	0.032	1.5	128
C600Rif/pIP1206	0	1.5	0.25	0.094	0.032	0.032	0.094	2	128
	+NMP	0.064	0.016	0.064	0.016	0.016	0.032	1.5	64
	+PAβN	0.047	0.008	0.032	0.012	0.012	0.006	1.5	64
TOP10	0	0.032	0.003	0.023	0.003	0.006	0.004	1.5	128
	+NMP	0.016	0.002	0.016	0.002	0.002	0.002	1	64
	+PAβN	0.016	0.002	0.016	0.002	0.002	0.002	1	64
TOP10/pIP1206	0	0.38	0.023	0.023	0.003	0.006	0.006	1.5	128
	+NMP	0.016	0.003	0.016	0.002	0.002	0.002	1	64
	+PAβN	0.016	0.002	0.016	0.002	0.002	0.002	1	64
TOP10/pAT851	0	0.19	0.016	0.023	0.003	0.008	0.004	1.5	128
	+NMP	0.016	0.002	0.016	0.002	0.002	0.002	1	64
	+PAβN	0.016	0.002	0.016	0.002	0.002	0.002	1	128

<sup>a</sup> CIP, ciprofloxacin; EBR, ethidium bromide; GAT, gatifloxacin; LVX, levofloxacin; MXF, moxifloxacin; NAL, nalidixic acid; NOR, norfloxacin; PFX, pefloxacin. MICs of fluoroquinolones and EBR were determined by E-test and agar dilution, respectively.

<sup>b</sup> NMP used at 40 μg/ml; PAβN used at 50 μg/ml.

ducers. Methylation at this position mediates aminoglycoside resistance by diminishing the affinity of the ribosome for gentamicin (10).

**Characterization of fluoroquinolone resistance.** The MICs of fluoroquinolones for transconjugant BM4650 (*E. coli* C600Rif/pIP1206) were determined by Etest (Table 1 and data not shown). A 10-fold or greater increase in the level of resistance to the hydrophilic quinolones, norfloxacin and ciprofloxacin, was observed against BM4650 relative to its plasmid-free counterpart. However, the increase in the MICs of norfloxacin and ciprofloxacin did not lead to a change in clinical categorization. By contrast, the MICs of relatively hydrophilic (pefloxacin, sparfloxacin, gatifloxacin, levofloxacin, and moxifloxacin) and hydrophobic (nalidixic acid) molecules were not significantly altered. In the presence of the NMP or PAβN efflux pump inhibitors, a 10-fold decrease in the level of resistance to norfloxacin and ciprofloxacin was observed (Table 1 and data not shown). These data suggest that efflux was responsible for resistance.

**Sequence analysis of the fluoroquinolone resistance gene and of the deduced protein.** Comparison of the nucleotide sequences obtained after shotgun cloning of pIP1206 DNA with those in the GenBank data library revealed no identity with genes for efflux pumps conferring fluoroquinolone resistance. BLASTX, which allows searching for regions of local similarity between translated query sequences versus protein sequences in the database, resulted in the identification of three open reading frames (ORFs), ORF1 to -3, in a 6,150-bp contig. The deduced sequence of ORF1 was found to be almost identical to that of a transposase-like protein in *Salmonella enterica* serovar Typhimurium (99% identity over 480 amino acids; GenPept accession number AAK02053). The sequence predicted from ORF2 was homologous to that of proteins belonging to the MFS from *Polaromonas* sp. (58% identity over 245 amino acids; GenPept accession number YP\_

548216) and from *Nitrosomonas eutropha* (55% identity over 230 amino acids; GenPept accession number YP\_747938). The translated sequence of ORF3 was identical to that of a class 1 integron integrase in various microorganisms (100% identity over 227 amino acids; GenPept accession number AAK02045) (Fig. 2).

Within ORF2, a putative GGAAG ribosome binding site (positions 4993 to 4989) was found 8 nucleotides upstream from an ATG initiation codon (position 4980) leading to a 1,536-bp putative coding sequence. The putative fluoroquinolone efflux gene, named *qepA* for quinolone efflux pump, specified a protein with a calculated mass of 53,080 Da.

The 511-amino-acid deduced QepA protein showed homology with various 14-transmembrane-segment (14-TMS) putative proton-dependent MFS transporters (Table 2 and Fig. 3) (19). These include SgcB from *Streptomyces globisporus* C-1027 (GenPept accession number AAL06672), Lic from *Leptospira interrogans* L1-130 (GenPept accession number YP\_000642), Nfa from *Nocardia farcinica* IFM 10152 (GenPept accession number YP\_118761), Neut from *N. eutropha* C91 (GenPept accession number YP\_747938), and Bpro from *Polaromonas* sp. strain JS666 (GenPept accession number YP\_548216). The

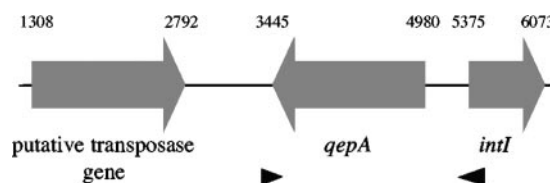


FIG. 2. Schematic representation of the flanking regions of the *qepA* gene. The gray arrows indicate the direction of transcription. The arrowheads represent oligodeoxynucleotides MFS8 and MFS9, used to generate pAT851 (pCR2.1Ω*qepA*). The coordinates correspond to GenBank accession number EF150886.



TABLE 2. Amino acid identities and similarities between 14-TMS MFS efflux proteins

Efflux protein (species) <sup>a</sup>	% Amino acid identity or similarity <sup>b</sup>					
	QepA	Bpro	Neut	Nfa	Lic	SgcB
QepA ( <i>E. coli</i> )		56.3	51.3	50.1	48.3	44.9
Bpro ( <i>Polaromonas</i> sp.)	70.3		54.0	52.7	60.3	43.1
Neut ( <i>N. eutropha</i> )	65.4	67.2		44.2	47.5	39.5
Nfa ( <i>N. farcinica</i> )	64.2	69.1	63.1		48.8	41.9
Lic ( <i>L. interrogans</i> )	65.6	75.6	65.9	68.3		43.5
SgcB ( <i>S. globisporus</i> )	60.9	60.5	57.6	59.1	59.8	

<sup>a</sup> The MFS efflux proteins were QepA from *E. coli* 1450 (this study and GenBank accession number EF150886), Bpro from *Polaromonas* sp. JS666 (GenPept accession number YP\_548216), Neut from *N. eutropha* C91 (GenPept accession number YP\_747938), Nfa from *N. farcinica* IFM 10152 (GenPept accession number YP\_118761), Lic from *L. interrogans* L1-130 (GenPept accession number YP\_000642), SgcB from *S. globisporus* C-1027 (GenPept accession number AAL06672).

<sup>b</sup> Percent amino acid identity values are presented in the upper half (above the diagonal space), and percent similarity values are given in the lower half (below the diagonal space).

levels of identity of QepA with these proteins varied from 45 to 56%, and the levels of similarity were between 61 and 70% (Table 2). TMS prediction with the TMHMM program indicated that the 14 TMSs of QepA were at positions similar to those in other 14-TMS proton-dependent efflux pumps (19), with the N and C termini located in the cytoplasm. The translocase consensus sequence (motif A), located in the loop between TMS 2 and TMS 3, and the drug extrusion consensus sequence (motif C) at the end of TMS 5 were conserved in all six sequences. Other motifs, in particular, D1, E, F, and H, which are exclusive to the 14-TMS cluster, confirmed that QepA is a new member of this family (Fig. 3) (19).

**Resistance conferred by the *qepA* gene.** A 2,095-bp fragment corresponding to the structural gene for the QepA efflux pump and its flanking regions was amplified by PCR with primers

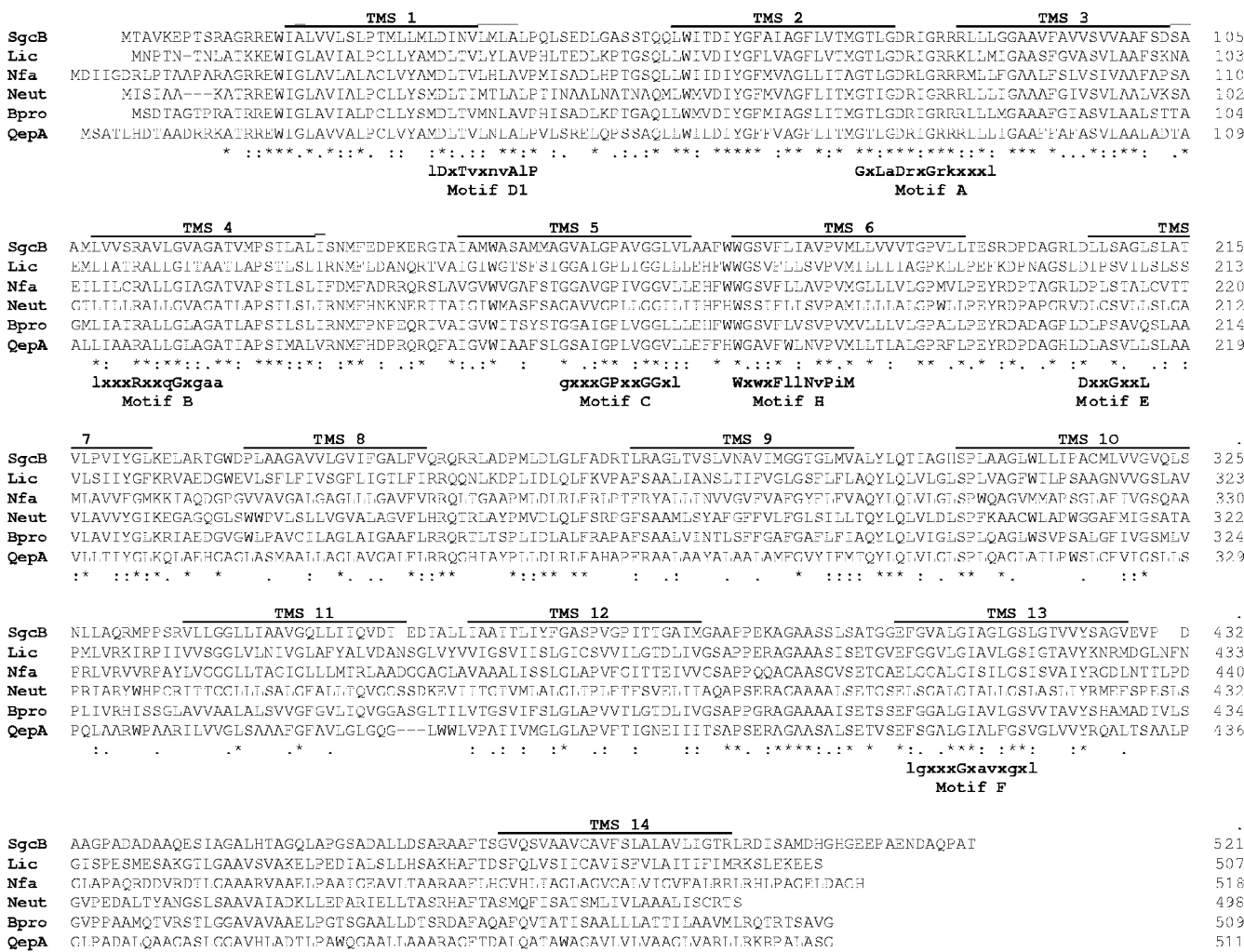


FIG. 3. Alignment of the QepA protein from *E. coli* 1450 with the sequences of 14-TMS MFS proteins. SgcB, from *S. globisporus* C-1027 (GenPept accession number AAL06672); Lic, from *L. interrogans* L1-130 (GenPept accession number YP\_000642); Nfa, from *N. farcinica* IFM 10152 (GenPept accession number YP\_118761); Neut, from *N. eutropha* C91 (GenPept accession number YP\_747938); Bpro, from *Polaromonas* sp. strain JS666 (GenPept accession number YP\_548216); and Qep, from *E. coli* 1450 (this study and GenBank accession number EF150886). Identical amino acids are indicated by a star, weakly conserved groups by dots, and strongly conserved groups by double dots. The horizontal lines above the alignment indicate the locations of the 14 TMSs (1 to 14). Highly conserved motifs (motifs A, B, C, D1, H, E, and F) are displayed below the alignment: x is any amino acid, a capital letter indicates that the amino acid occurs in >70% of examined sequences, and a lowercase letter indicates that the amino acid occurs in <40% of examined sequences (19).

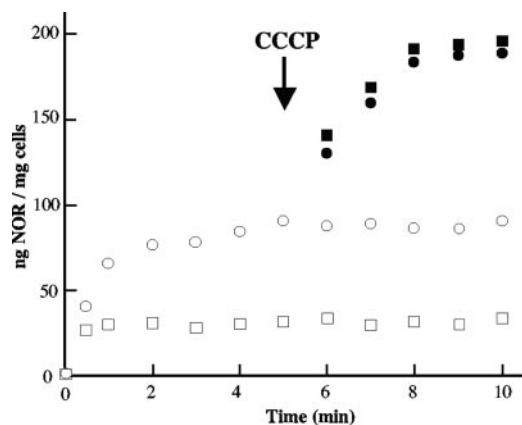


FIG. 4. Accumulation of norfloxacin (NOR) by *E. coli* cells carrying pCR2.1 (circles) or pAT851 (pCR2.1 $\Omega$ qepA) (squares), without (open symbols) or with (closed symbols) CCCP. Addition of CCCP (100  $\mu$ M) is indicated by an arrow.

MFS8 (positions 3405 to 3422), 5'-AGCAGCGCGCTGAAT CCA-3', and MFS9 (positions 5499 to 5482), 5'-CGAACCCA GTGGACATAA-3', and cloned in pCR2.1 to generate pAT851 (pCR2.1 $\Omega$ qepA). The recombinant plasmid conferred on *E. coli* TOP10 and AG100A a fivefold or greater increase in resistance to the hydrophilic fluoroquinolones norfloxacin and ciprofloxacin only. In the presence of NMP or PA $\beta$ N efflux pump inhibitors, the MICs of norfloxacin and ciprofloxacin were reduced at least sixfold (Table 1). The MICs of other antimicrobial agents (chloramphenicol, erythromycin, tetracycline, minocycline, tigecycline, and rifampin) and of dyes (acridine orange, acriflavine, and ethidium bromide) known to be substrates for efflux pumps remained unchanged against the strain harboring pAT851 (pCR2.1 $\Omega$ qepA) (Table 1 and data not shown). Taken together, these results suggested that QepA acts through drug-specific efflux.

To confirm that the difference in norfloxacin susceptibility between *E. coli* strains carrying pCR2.1 or pAT851 (pCR2.1 $\Omega$ qepA) was due to an efflux mechanism, the accumulation of norfloxacin was measured and found to be approximately three times lower in *E. coli* carrying pAT851 than in the control strain (Fig. 4). Dissipation of the membrane proton motive force by addition of the protonophore CCCP increased norfloxacin accumulation in both strains, resulting in similar concentrations.

The 72% overall guanine-plus-cytosine (%GC) content of the qepA gene significantly differed from that of the flanking DNA (51% upstream and 60% downstream, respectively). This observation suggests a recent acquisition by plasmid pIP1206 of a gene from a microorganism with a high %GC content. However, because of a lack of sequences with a high degree of identity to qepA in the data banks, the actual progenitor of the resistance determinant remains unknown. The %GCs of genes for efflux pumps in antibiotic-producing streptomycetes, which have to protect themselves against suicide by the products of their secondary metabolism, range from 64% to 72% (11). This is in contrast with the base composition of the genomes of members of the family Enterobacteriaceae, which is ca. 50%. These observations favor an origin in actinomycetes for qepA, which is rather surprising in view of the

fact that quinolones are synthetic antibiotics. It is therefore likely that we did not test the preferred substrate for the QepA pump.

In the conjugative plasmid pIP1206, qepA is flanked by genes known to be associated with the movement of genetic elements and is also physically linked to the rmtB and bla<sub>TEM-1</sub> resistance determinants. This genetic organization could further enhance the dissemination of qepA among human pathogens, in particular by coselection by aminoglycosides and  $\beta$ -lactams. In fact, a plasmid-mediated 14-TMS fluoroquinolone efflux pump has been reported recently in an aminoglycoside-resistant *E. coli* strain in Japan (25).

The qepA gene, together with the qnr family and aac(6')-Ib-cr, is the third recently detected plasmid-borne determinant of resistance to the fluoroquinolones. These genes confer only low-level resistance, but their presence could potentially facilitate evolution of the bacterial host toward higher levels of resistance by mutational alterations in the target type II topoisomerases.

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