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oqxAB Encoding a Multidrug Efflux Pump in Human Clinical Isolates of *Enterobacteriaceae*▽

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The genes for multidrug efflux pump OqxAB, which is active on fluoroquinolones, were found in human clinical isolates on a plasmid in *Escherichia coli* and on the chromosome of *Klebsiella pneumoniae*. IS*26*-like sequences flanked the plasmid-mediated *oqxAB* genes, suggesting that they had been mobilized as part of a composite transposon.

Plasmid-borne genes conferring quinolone resistance have been increasingly recognized (7, 10). Recently a plasmid-encoded efflux pump, OqxAB, conferring resistance to the quinoxaline-di-*N*-oxide olaquindox, which has been used as a growth promoter in pigs, was discovered in *Escherichia coli* isolates of porcine origin in Denmark and Sweden (4–6). OqxAB was encoded by the genes *oqxA* and *oqxB* located on a 52-kb conjugative plasmid designated pOLA52 and conferred resistance to multiple agents, including fluoroquinolones (4, 9). We have investigated the prevalence of this plasmid-encoded multidrug efflux pump in clinical isolates of *Enterobacteriaceae* and have for the first time identified an *oqxAB*-encoding plasmid in an *E. coli* isolate of human origin.

Isolates were from the collection of blood isolates from Seoul National University Hospital collected from 1998 to 2006. The same set of isolates was previously surveyed for other plasmid-mediated quinolone resistance (PMQR) genes (8). A total of 461 clinical isolates were screened by PCR for the *oqxA* gene. Isolates positive for *oqxA* were also tested for *oqxB*, and strains positive for both genes were confirmed by sequencing of the PCR products. The primers used are shown in Table 1.

One (0.4%) of 261 *E. coli* isolates, 3 (4.6%) of 65 *Enterobacter cloacae* isolates, and 100 (74.1%) of 135 *Klebsiella pneumoniae* isolates were provisionally classified as positive for both *oqxA* and *oqxB*. The *oqxAB*-positive *E. coli* was isolated from the blood of a patient in 1999. A BLAST search of the nucleotide sequence similarity of the *oqxB* PCR products obtained from the three *E. cloacae* isolates gave identities of only 88% (399/453) with pOLA52 (GenBank accession number EU370913) and 86% (394/454) with the hydrophobe/amphiphile efflux-1 (HAE1) family transporter of *Enterobacter* sp. strain 638 (GenBank accession number CP000653). There was, however, substantial similarity between the complete nucleotide sequences of the tandem *oqxA* and *oqxB* genes from *E. coli* 1-12 (GenBank accession number GQ120634; 99.5% and 99.0%, respectively), *K. pneumoniae* 4-39 (GenBank accession number FJ975560; 98.2% and 99.0%, respectively), and *K. pneumoniae* 5-80 (GenBank accession number FJ975561; 99.4% and 98.9%, respectively) relative to those of pOLA52 and the chromosomal genes in *K. pneumoniae* MGH78578 (GenBank accession number NC009648). Since the *oqxAB* genes appear to be chromosomal in *K. pneumoniae* (3, 9), *E. coli* 1-12 was the most likely candidate to contain an *oqxAB*-encoding plasmid (Table 2).

To test for the plasmid location of *oqxAB*, plasmid DNAs were obtained using a plasmid midi kit (Qiagen, Valencia, CA) and hybridized with a horseradish peroxidase-labeled *oqxB* probe as previously described (12). While the seven *E. coli* strains, one *E. cloacae* strain, and one *K. pneumoniae* strain tested all contained one or more plasmids, only the plasmid from *E. coli* 1-12 hybridized to the *oqxB* probe, and this plasmid was estimated to be more than 100 kb in size (Fig. 1) (Table 2). For *K. pneumoniae*, whole-cell DNA was also used for hybridization, and strong signals were seen with bands comigrating with chromosomal DNA for all four PCR-positive strains tested. Of the four repeatedly PCR-negative *K. pneumoniae* strains (1-68, 2-54, 3-45, and 5-22) tested by Southern hybridization, two had no signal with the *oqxB* probe, and two had a weak signal.

To determine whether the presence of *oqxAB* genes was associated with resistance to olaquindox, we determined MICs with olaquindox (MP Biomedicals, Inc., Solon, OH) (5) for nine *E. coli* (including *oqxAB*-positive strain 1-12), six *E. cloacae* (three positive and three negative for *oqxAB* by PCR), and 10 *K. pneumoniae* (6 positive and 4 negative for *oqxAB*) strains. In *E. coli* and *E. cloacae* strains and all but two *K. pneumoniae* strains, the presence of *oqxAB* or related genes by PCR correlated with olaquindox MICs of ≥128 μg/ml. Two *oqxAB*-positive *K. pneumoniae* strains, however, had MICs of only 16 μg/ml (Table 2). Ciprofloxacin MICs also varied among the tested strains. These strains lacked other PMQR genes, with the exception of one strain positive for *qepA* and

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TABLE 1. Primers used in this study

Gene	Primer	Sequence (5′→3′)	Size of the amplified product (bp)
For PCR[a]			
oqxA	oqxAF	CTCGGCGCGATGATGCT	392
	oqxAR	CCACTCTTCACGGGAGACGA	
oqxB	oqxBs	TTCTCCCCCGGCGGGAAGTAC	512
	oqxBa2	CTCGGCCATTTTGGCGCGTA	
For real-time RT-PCR			
adk	adkKF	ATGCGTATTATTCTGCTTGGCGC	105
	adkKR	CAGCATATCGCCGGTGGAGAT	
oqxB	oqxBKF	TCCTGATCTCCATTAACGCCCA	131
	oqxBKR	ACCGGAACCCATCTCGATGC	

[a] The PCR conditions for *oqxA* were 94°C for 45 s, 57°C for 45 s, and 68°C for 60 s with a cycle number of 34, and those for *oqxB* were 94°C for 45 s, 64°C for 45 s, and 72°C for 60 s with a cycle number of 32.

two positive for *aac(6′)-Ib-cr*. *E. coli* 1-12 had a ciprofloxacin MIC of >32 μg/ml, but four other *E. coli* strains and one *K. pneumoniae* strain negative for *oqxAB* did as well, likely reflecting the presence of additional chromosomal mutations. In *K. pneumoniae* strains, the four strains with higher olaquindox MICs had higher MICs of ciprofloxacin, but there were no differences in the low MICs of ciprofloxacin for strains of *E. cloacae* that were positive and negative for *oqxAB* by PCR.

To determine whether *oqxAB* was transferable from *E. coli* 1-12, conjugation experiments were carried out in Luria-Bertani (LB) broth or on filters with azide-resistant (Az^r) *E. coli* J53 as the recipient at 37°C (12). Transconjugants were selected on LB agar plates containing ampicillin (100 μg/ml), chloramphenicol (25 or 50 μg/ml), olaquindox (32 or 64 μg/ml), or trimethoprim (2 μg/ml), depending on the antibiogram of the donor strain, and sodium azide (100 μg/ml) for counterselection. In addition, plasmids isolated from *E. coli* 1-12 were transformed into electrocompetent *E. coli* DH10B and plated onto LB plates containing ampicillin, chloramphenicol, or olaquindox. Although resistance to ampicillin was transferred, no direct transfer or cotransfer of *oqxAB* was found by either conjugation or electroporation. This finding suggests that the *oqxB*-hybridizing plasmid was nonconjugative under these conditions. Similarly, the *E. cloacae* strains that were positive for *oqxAB* by PCR and the six *K. pneumoniae oqxAB*-positive strains yielded no transconjugants.

TABLE 2. Summary of the characteristics of selected strains of *E. coli*, *E. cloacae*, and *K. pneumoniae*[d]

Strain	*oqxA* and *oqxB* PCR result	PMQR genes	MIC[a] (μg/ml)		Southern blotting result for *oqxB*[c]
			Ciprofloxacin	Olaquindox	
E. coli					
ATCC 25922	−	−	0.008	8–16	N.D.
J53 Az^r	−	−	0.012	8–16	−
1-12	+	−	>32	256	+
4-67	−	−	0.125	16	−
4-69	−	−	>32	16	−
4-78	−	−	>32	16	−
5-56	−	−	>32	16	−
5-65	−	−	0.75	16	−
5-59	−	*qepA*	>32	16	−
E. cloacae					
1-26	+[b]	−	0.047	128	−
1-39	+[b]	−	0.032	128	N.D.
1-40	+[b]	−	0.047	128	N.D.
1-3	−	−	0.047	64	N.D.
4-11	−	−	0.047	32	N.D.
6-4	−	−	0.047	16	N.D.
K. pneumoniae					
3-51	+	−	0.047	16	+
4-39	+	−	0.064	16	+
5-80	+	−	3.0	>256	+
6-49	+	−	>32	>256	+
4-13	+	*aac(6′)-Ib-cr*	>32	>256	N.D.
4-38	+	*aac(6′)-Ib-cr*	>32	>256	−
1-68	−	−	0.047	8	+/−
2-54	−	−	0.023	8	−
3-45	−	−	0.023	16	+/−
5-22	−	−	0.064	8	−

[a] Ciprofloxacin MICs were determined by Etest and olaquindox MIC by broth dilution using LB broth.
[b] PCR positive for *oqxAB*; the PCR product was 88% identical to *oqxAB* of pOLA52.
[c] N.D., not done.
[d] +, positive; −, negative; +/−, weak positive.

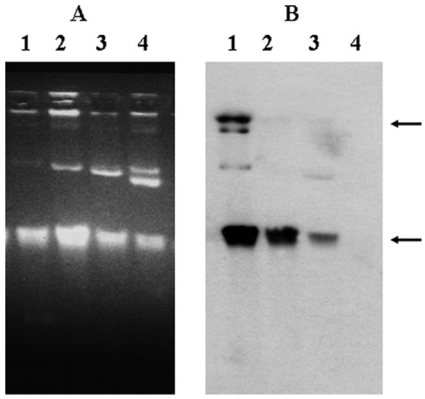

FIG. 1. Gel electrophoresis (A) and Southern hybridization (B) of plasmid DNA preparations. Lanes: 1, *E. coli* 1-12; 2, *E. cloacae* 1-26; 3, *K. pneumoniae* 4-38; and 4, *E. coli* 4-67. Upper arrow indicates plasmid DNA; lower arrow indicates the location of chromosomal DNA and/or sheared plasmid DNA.

We sequenced flanking DNA and the entirety of *oqxAB* in *E. coli* 1-12 with a series of outward-facing primers, starting from both sides of each PCR product of *oqxA* and *oqxB*, using an inverse PCR strategy (11). We digested DNA with NcoI or NgoMIV (New England Biolabs, Ipswich, MA) and ligated the digested DNA with T4 DNA ligase (New England Biolabs, Ipswich, MA). We then performed inverse PCRs using the primers designed from both *oqxA* (5′-AACCTCGTCTCCCGTGAAGAGTGG and 5′-TGAACGCTCTCCACCGCTTCAA) and *oqxB* (5′-CAGCTCAACAATAAGGATGCGGTC and 5′-GGAGATCAGGAAATCGCTCTCCTG), using PCR conditions of 95°C for 10 min, followed by 30 cycles of 4°C for 1 min, 55°C for 2 min, and 72°C for 4 min, followed by a final extension at 72°C for 10 min. A 6,027-bp DNA segment containing the *oqxAB* genes was found to be flanked by IS*26*-like sequences and to match completely the sequence surrounding *oqxAB* in pOLA52 (9) (bases 46,312 to 51,602 and 1 to 736 [GenBank accession no. EU370913]). Thus, the *oqxAB* genes in *E. coli* 1-12 appear to be located on a composite transposon previously named Tn*6010* (9).

Since the *oqxAB*-positive isolates of *K. pneumoniae* differed in their levels of resistance to olaquindox and ciprofloxacin, we compared the relative expression levels of the *oqxAB* genes in the four *K. pneumoniae* isolates without other PMQR genes.

TABLE 3. Expression of the *oqxB* gene in *K. pneumoniae* strains with or without the olaquindox resistance phenotype[a]

Strain	Mean relative expression	SEM
3-51	1.0	0
4-39	1.0	0.2
5-80	21.7	11.9
6-49	11.4	6.7

[a] Each strain was grown in LB broth at 37°C without antibiotics. The relative expression of *oqxB* was normalized with an internal control for the level of expression of *adk* (1). The relative expression of the *oqxB* gene was calculated by the $\Delta\Delta C_T$ method, in which the amount of target cDNA, normalized to *adk* and relative to an in vitro calibrator, is given by the variable $2^{-\Delta\Delta CT}$, where C_T is the cycle number of the detection threshold. ΔC_T (the difference between C_T values of *oqxB* and *adk*) of strains 3-51 and 4-39 ranged from 3.17 to 5.05, and $\Delta\Delta C_T$ was calculated from ΔC_T of strain 3-51 as the baseline. Averages and standard errors of the mean (SEM) were calculated from the results of two independent experiments.

Each strain was grown in LB broth at 37°C, cells were collected at an optical density at 600 nm of ~0.5 (after 2 to 2.5 h), and total RNA was isolated with an RNeasy mini kit (Qiagen, Valencia, CA). cDNA synthesis and quantitative PCR amplification were conducted as previously described (2). The relative levels of expression of the *oqxAB* genes correlated with the level of olaquindox resistance (Table 3), suggesting that different levels of chromosomal gene expression may account for the differences in resistance and may contribute in part to the elevated MICs of ciprofloxacin. No differences in the sequences 5′ of *oqxA* were found between strains 4-39 and 5-80, which differed 20-fold in levels of *oqxB* transcripts. Thus, the increased expression of *oqxB* in the two strains appears not to be due to mutation in a putative promoter, but might relate to differences in other as-yet-undefined regulatory elements in these strains.

This is the first report of the presence of an *oqxAB*-containing plasmid in a human isolate of *E. coli*. Although transfer of the plasmid was not successful, Southern blotting for *oqxB* indicated that these genes were located on a plasmid, rather than the chromosome. Considering that the *oqxAB* genes are chromosomally located in *K. pneumoniae* and highly prevalent in clinical isolates, the plasmid containing *oqxAB* seems to have arisen by capture from the *K. pneumoniae* genome, which may be a reservoir for this antibiotic resistance determinant (9). The natural function of *oqxAB* remains unknown.

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