Characterization of qnrB-Like Genes in Citrobacter Species of the American Type Culture Collection

Tomoo Saga,a,b Stefana Sabtcheva,b Kotaro Mitsutake,b Yoshikazu Ishii,a Kazuhiro Tateda,a Keizo Yamaguchi,a Kazuhiro Kaku=a
Department of Microbiology and Infectious Diseases, Toho University School of Medicine, Oota-ku, Tokyo, Japan; Department of Infection Control and Molecular Diagnostics, Tohoku University School of Medicine, Aoba-ku, Sendai, Japan

Among five American Type Culture Collection (ATCC) Citrobacter strains, qnrB60 in Citrobacter freundii ATCC 6879, an isolate from the preantibiotic era, and qnrB61 in Citrobacter braakii ATCC 51113T, a type strain belonging to the C. freundii complex, were identified. Meanwhile, a truncated qnrB-like pseudogene was identified in C. freundii ATCC 8090T and ATCC 43864. No qnrB-like sequence was found in Citrobacter koseri ATCC 27028T. These findings underscore the close relationship between this species and qnrB.

An increasing number of plasmid-mediated quinolone resistance (PMQR) determinants have been reported (1, 2). In most studies, PMQR determinants have been found among multiple-drug-resistant bacteria, especially among extended-spectrum β-lactamase producers. Despite its only recent discovery, qnrB is one of the most prevalent PMQR determinants among clinical isolates of Enterobacteriaceae (3–7). Interestingly, the distribution of qnrB is uneven among species; most new variants of qnrB have been found in Citrobacter spp., and the prevalence of qnrB seems high among certain populations of Citrobacter spp. strains, a substantial part of which were not multidrug resistant (8). One possible explanation is that qnrB was originally present among some populations of Citrobacter bacteria irrespective of quinolone exposure. More recently, the association between qnrB and clinical isolates of Citrobacter spp. has been well documented by Jacoby et al. (8). In the present study, to clarify this linkage more generally and robustly, reference Citrobacter strains of the American Type Culture Collection (ATCC), including type strains, nonclinical isolates, and a strain from the preantibiotic era, were subjected to detection and characterization of the qnrB-like gene (9–11).

(This work was presented in part at the 49th Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco, CA, 2009.)

Five Citrobacter strains of the ATCC, including three type strains, were subjected to qnr detection by PCR, i.e., Citrobacter freundii ATCC 8090T, C. freundii ATCC 43864, C. freundii ATCC 6879, Citrobacter braakii ATCC 51113T, and Citrobacter koseri ATCC 27028T (see Table S1 in the supplemental material). Two strains, namely, C. freundii ATCC 6879 and C. braakii ATCC 51113T, were positive for qnrB with two different primer pairs. qnrA and qnrS were not amplified in any strain. In order to perform cloning of qnrB, a BamHI-digested genomic DNA fragment containing the qnrB-like gene and BamHI-digested, bacterial alkaline phosphatase (BAP)-treated pUC119 (TaKaRa Bio Inc., Otsu, Japan) were ligated with TaKaRa Ligation Mighty Mix (TaKaRa Bio Inc.) and chemically transformed into Escherichia coli TOP10 (Invitrogen, Carlsbad, CA). Transformants were selected on LB-agarose plates containing 0.016 μg/ml of levofloxacin to screen for strains with elevated quinolone resistance and thereafter on plates containing 100 μg/ml of ampicillin (Sigma-Aldrich, St. Louis, MO) to confirm that the resultant clones were truly transformants. We successfully cloned a 5,513-bp DNA fragment containing qnrB61 of C. braakii ATCC 51113T. qnrB61 was 645 bp, and its upstream and downstream regions were the psp gene cluster and the sap gene cluster, respectively (Fig. 1). qnrB61 differed from QnrB10 by one amino acid (G17S). By I-CeuI digestion, followed by pulsed-field gel electrophoresis and Southern hybridization (12), qnrB61 was shown to be located on the chromosome, but not on the plasmids, of C. braakii ATCC 51113T (data not shown). Our attempt to clone the qnrB-like gene of C. freundii ATCC 6879 was unsuccessful.

PCR amplification and sequencing of the region between psfF and sapA confirmed that C. freundii ATCC 6879 harbors a full-length qnrB gene (qnrB60) and that C. braakii ATCC 51113T harbors qnrB61. QnrB60 in C. freundii ATCC 6879 was found to differ by one amino acid from QnrB38 (A202V). The promoter region and putative SOS box of the upstream region of qnrB60 and qnrB61 were intact. In contrast, the truncated qnrB-like pseudogenes in C. freundii ATCC 8090T and C. freundii ATCC 43864 (99.6% identity) were found between psfF and sapA; these pseudogenes contained the last 285 bp of the qnrB-like nucleotide sequence (97% identical to qnrB19 and qnrB36) but lacked the first 360 bp of the putative open reading frame in addition to the upstream region, including the promoter and SOS box (Fig. 1). In contrast, there was no nucleotide sequence homologous to qnrB in C. koseri ATCC 27028T.

To estimate the contribution of the qnrB-like gene to reduction of quinolone susceptibility in E. coli, we constructed vectors expressing each qnrB-like gene under the control of the same promoter and compared the drug susceptibility of transformed E. coli TOP10. A T vector was constructed by PCR with primers pTV-T-EcoRV-F and pTV-T-EcoRV-R (see Table S1 in the supplemental material) for the transformation of E. coli TOP10. (See Table S1 in the supplemental material.)
Acquired qnrB expression is known to be regulated by the SOS response in E. coli (14, 15). Therefore, regulation of chromosomal qnrB in Citrobacter spp. was examined. C. braakii ATCC 51113T was incubated at 35°C with shaking in LB broth. Full-growth medium was diluted by adding a 40-fold volume of fresh medium. After 1 h of incubation, levofloxacin at 1× MIC (0.06 µg/ml), 0.5× MIC (0.03 µg/ml), or 0.25× MIC (0.015 µg/ml) was added, and after an additional 4 h of incubation, 0.5 ml of broth was harvested. Reverse transcriptase PCR was performed with a Qiagen RNeasy kit, RNAprotect bacterial reagents, a DNase kit (Qia-gen, Valencia, CA), a cDNA High reverse transcriptase kit (Ap-ploed Biosystems, Foster City, CA), and SYBR green ER quantitative PCR Supermix for ABI Prism (Invitrogen), and the product was analyzed with the ABI Prism 7000 sequence detection system (Applied Biosystems). As expected, expression of qnrB61 was increased in parallel with the upregulation of recA and lexA, which are key effectors of the SOS response (Fig. 2). This result was consistent with the presence of an intact SOS box upstream of qnrB61 in C. braakii ATCC 51113T.

As quinolone is a synthetic antimicrobial agent that was introduced into the clinical setting in the early 1960s, the quinolone resistance determinant was not expected to be prevalent in nature before the development and practical use of quinolones. Our findings in the present study, however, indicated that there should be a revision of this framework; a “functional” qnrB61 gene was identified on the chromosome of the type strain of C. braakii, ATCC 51113T, which is a member of the C. freundii complex and was isolated from a snake (http://www.atcc.org/) (9). qnrB60 was also found in C. freundii ATCC 6879, which was an isolate from milk set for cottage cheese before 1932 in the preantibiotic era, long before the synthesis of quinolones (11). Moreover, ATCC 8090T, a type strain of C. freundii isolated before 1931, harbored a truncated qnrB pseudogene (10). Although the psp and sap gene clusters have been identified in a wide variety of chromosomes of Enterobacteriaceae, the presence of a qnrB-like sequence between these clusters seems unusual (16). Although our results are not conclusive in themselves, others have also suggested that there might be a relationship between the C. freundii complex and quinolone resistance: for example, Jacoby et al. demonstrated the presence of qnrB or its pseudogene in clinical C. freundii complex strains (8). The integrative hypothesis is that a progenitor of the C. freundii complex, but not of other

---

**FIG 1** Genetic map of the region between the psp and sap gene clusters of Citrobacter ATCC strains. Each underlined value is the length (bp) of the qnr-like gene/pseudogene, and its upstream/downstream environments are separated by vertical black bars. In C. koseri ATCC 27028T, truncated putative open reading frame CKO_01383, but no qnr-like gene/pseudogene, was identified between pspF and sapA.
members of the family Enterobacteriaceae, including *C. koseri*, might have acquired the qnrB-like gene in its chromosomal region between the *psp* and *sap* gene clusters in the past. Thereafter, although some *C. freundii* complex strains have preserved a functional qnrB-like gene to date, others have presumably lost it by deletion, leaving the pseudogene as a trace. Although the transcription of qnrB61 in the chromosome of *C. braakii* ATCC 51113<sup>3</sup> was increased, presumably via the SOS response, by exposure to fluoroquinolone (and this agent might potentially facilitate the selection of qnr<sup>-</sup>-positive strains), the physiological role of qnr would be essentially independent of the presence of quinolone agents, as suggested by a bioinformatic approach (17).

Another finding regarding the function of Qnr in the present study was that both termini of qnrB61 seem to be necessary to confer quinolone resistance on *E. coli*. This result seems consistent with the study of Guo et al. in which the deletion of only around 10 amino acids from each terminus of QnrA1 abolished quinolone resistance (18).

In conclusion, our results with reference ATCC strains strengthen the relationship between qnrB and *Citrobacter* spp. more generally and robustly. The physiological role of Qnr in bacteria, as well as the actual role of the development of quinolone resistance, remains to be elucidated by further studies.

**Nucleotide sequence accession numbers.** The nucleotide sequences determined in this study have been deposited in the DNA databases and assigned accession numbers AB734052 to AB734056.

**ACKNOWLEDGMENTS**

We thank George Jacoby for his management and suggestion concerning qnr nomenclature. We thank the laboratory members for their technical and scientific assistance.

Part of this work was supported by Grant-in-Aid for Young Scientists (B) 20790712 from the Ministry of Education, Culture, Sports, Science,
and Technology, Japan; by Toho Project research grants 20-01 and 21-5 from the Toho University School of Medicine to T.S.; and by Grant-in-Aid for Scientific Research 17-05764 from the Japan Society for the Promotion of Science to S.S.

REFERENCES


