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Complex Class 1 Integron Carrying *qnrB62* and bla_{VIM-2} in a *Citrobacter freundii* Clinical Isolate

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The coexistence of *qnrB62* and bla_{VIM-2} was detected in a *Citrobacter* clinical isolate. The reduced fluoroquinolone susceptibility is attributable to *qnrB62*, mutations of quinolone-resistance-determining regions, and an efflux pump or pumps. The genetic context surrounding chromosomal *qnrB62* was a novel complex class 1 integron (In1184::IS*CR1*::*qnrB62*) containing a unique gene array (bla_{VIM-2}-*aacA4′-8-gucD*). An 18-nucleotide deletion at the 3′ end of the *pspA* gene [*pspA*(Δ*18*)], upstream of *qnrB62*, and an inverted repeat region (IRR2) were detected in In1184::IS*CR1*::*qnrB62*, indicating past transposition events.

The spread of carbapenem-resistant *Enterobacteriaceae* is a significant threat to public health (1, 2). The *qnrB* family is represented by 80 different alleles (http://www.lahey.org/qnrStudies/), most of which originated from *Citrobacter* strains and spread to other *Enterobacteriaceae* species (3). The association of *qnrB* with extended-spectrum β-lactamase and plasmid-mediated AmpC β-lactamase genes has been reported and is of great concern. Furthermore, a KQ (*Klebsiella pneumoniae* carbapenemase [KPC] and QNR) element encoding a KPC and QnrB19 was reported (4). However, the coexistence of *qnrB* and bla_{VIM-2} has not yet been reported in the clinic.

We present here (i) the first report of the complex class 1 integron that simultaneously carries bla_{VIM-2} and a new *qnrB* allele (*qnrB62*) in a *Citrobacter* clinical isolate resistant to fluoroquinolones and carbapenems, (ii) other mechanisms responsible for fluoroquinolone resistance, and (iii) potential horizontal spread of the *qnrB62* (or bla_{VIM-2}) gene.

Citrobacter freundii 11-7F4560 was isolated from a urine sample collected from a kidney transplant patient at a tertiary care hospital in the Republic of Korea in 2011. The bacterial species was identified using the Vitek 2 system (bioMérieux) and confirmed by 16S rRNA sequencing. MICs were determined using by the agar dilution method as per the Clinical and Laboratory Standards Institute guidelines (5). *C. freundii* 11-7F456011 was resistant to all tested (fluoro)quinolones (nalidixic acid, ofloxacin, levofloxacin, and ciprofloxacin) (Table 1) and additionally resistant to carbapenems (MICs of 8 μg/ml for imipenem, 8 μg/ml for meropenem, and 4 μg/ml for ertapenem). This resistance was mediated by the bla_{VIM-2} gene as described below. In the *C. freundii* isolate, we investigated the mechanisms responsible for quinolone resistance.

PCR-based detection of plasmid-mediated quinolone resistance (PMQR) genes (*qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *qnrVC1*, *qnrVC4*, *aac6′-Ib-cr*, *qepA*, *oqxA*, and *oqxB*) and quinolone resistance-determining regions (QRDRs) of *gyrA*, *gyrB*, *parC*, and *parE* was performed as previously described (6–9). The positive-control strain for the *qnrB* gene was a *C. freundii* clinical isolate harboring *qnrB23* (8). The other 10 positive-control strains for the investigated PMQR genes were *Escherichia coli* TOP10 transformants harboring pHSG398 with each gene (i.e., *qnrA1* [accession no. AY070235], *qnrC* [EU917444], *qnrD1* [FJ228229], *qnrS1* [AB187515], *qnrVC1* [EU436855], *qnVC4* [GQ891757], *aac6′-Ib-cr* [DQ303918], *qepA* [NG_050459], *oqxA* [NG_048024], or *oqxB* [NG_048025]), which were chemically synthesized using the GeneArt gene synthesis kit (Life Technologies Korea LLC., Seoul, Republic of Korea). The PCR-based detection of PMQR genes identified only a novel variant (*qnrB62*) of the *qnrB* gene, which was assigned by the Lahey website (http://www.lahey.org/qnrStudies/). Amplicon sequencing analysis revealed a 681-bp open reading frame encoding a 226-amino-acid protein (QnrB62) with 99.6% amino acid sequence identity to the QnrB5 sequence (accession no. DQ303919) (10). In comparison with QnrB5, QnrB62 has one amino acid substitution (Ser198Asn; QnrB numbering system [11]).

To determine MICs of the QnrB62-harboring transformant, the PCR amplicon of the *qnrB62* gene was cloned into the vector pHSG398 (chloramphenicol resistant; TaKaRa), and the recombinant pHSG398-*qnrB62* plasmid was transformed into the *Escherichia coli* TOP10 host strain. The primers used for cloning of *qnrB62* were SacI-QnrB62-F (5′-ATA**GAGCTC**AATGACGCCATTACTGTATAAAACA-3′) and XbaI-QnrB62-R (5′-GAC**TCTAGA**CTAGCCAATAATCGCGATGC-3′), in which the restriction recognition sites are shown in boldface. To compare MICs of the *E. coli* TOP10 transformant harboring pHSG398-*qnrB62* with those of the transformant harboring pHSG398-*qnrB5*, the QnrB62N198S mutant (QnrB5 with the substitution of Asn198 with serine) was generated using a PCR-driven overlap extension method (12) with specific primers (QnrB62N198S-B [5′-GCCTGGTAGCTGTCTAACTT-3′] and QnrB62N198S-C [5′-AAGTTAGACAGCTACCAGGC-3′]). A decrease in (fluoro)quinolone susceptibility was observed with both transformants (TrfQnrB5

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TABLE 1 Characteristics of the *Citrobacter* isolate, *Escherichia coli* TOP10 transformants and host strain, MIC reference strain, plasmids, and primers used in this study

Strain	QRDR[a] mutations	MIC (μg/ml) of[b]:								
		PAβN	NAL	NAL + PAβN[c]	OFX	OFX + PAβN[c]	LVX	LVX + PAβN[c]	CIP	CIP + PAβN[c]
Citrobacter freundii 11–7F4560[d]	Thr83Ile (*gyrA*), Phe431Ser (*gyrB*), Ser80Ile (*parC*)	>256	>256	32	>32	4	32	2	32	8
E. coli[e]										
TrfQnrB62		>256	8	1	0.25	0.06	0.125	0.03	0.06	0.03
TrfQnrB5		>256	8	1	0.25	0.06	0.125	0.03	0.06	0.03
TOP10		>256	1	0.125	0.06	0.03	0.015	0.0075	0.0075	0.0075
ATCC 25922		>256	1	0.125	0.06	0.03	0.015	0.0075	0.0075	0.0075

[a] QRDR, quinolone resistance-determining region.
[b] PAβN, Phe-Arg-β-naphthylamide; NAL, nalidixic acid; OFX, ofloxacin; LVX; levofloxacin; CIP; ciprofloxacin.
[c] The PAβN concentration used was 64 μg/ml for all tested isolates/strains.
[d] Clinical isolate with the gene *qnrB62* in its chromosome.
[e] TrfQnrB62 and TrfQnrB5 are *E. coli* TOP10 transformants harboring pHSG398-*qnrB62* (pHSG398 containing *qnrB62* from *C. freundii* 11-7F4560) and pHSG398-*qnrB5* (pHSG398 containing *qnrB5* [$qnrB62_{N198S}$ mutated from pHSG398-*qnrB62*]), respectively. TOP10 is the host strain, and ATCC 25922 is the MIC reference strain.

and TrfQnrB62 in Table 1). This result suggests that the reduced quinolone susceptibility of the *C. freundii* isolate is attributable in part to *qnrB62*, which was comparable to the presence of *qnrB5* in the *Salmonella enterica* isolate (10). However, the MICs of the *C. freundii* isolate were higher than those of the *S. enterica* isolate (MICs of 32 μg/ml for nalidixic acid and 0.5 μg/ml for ciprofloxacin) (10). These differences between the two isolates were associated with two distinct mechanisms of quinolone resistance.

First, PCR-based detection of QRDR mutations identified a Thr83Ile substitution in GyrA, a Phe431Ser substitution in GyrB, and a Ser80Ile substitution in ParC (Table 1). The Thr83Ile and Ser80Ile substitutions are sufficient for decreased fluoroquinolone susceptibility in *Citrobacter* isolates and *E. coli* isolates, respectively (7, 13). However, Phe431Ser in GyrB is a novel substitution in the *Citrobacter* isolates, and the significance of this mutation is unclear. Second, the MICs of all tested (fluoro)quinolones varied by more than 4-fold in the presence of the efflux pump inhibitor Phe-Arg-β-naphthylamide (Table 1), suggesting variable contribution of an efflux pump or pumps in determining quinolone susceptibility. However, plasmid-mediated *qepA*, *oqxA*, and *oqxB* genes were not detected in *C. freundii* 11-7F456011, suggesting the involvement of chromosomal efflux pumps (e.g., intrinsic RND-type efflux pumps) other than QepA or OqxAB in the reduced quinolone susceptibility of the *C. freundii* isolate. Further study is needed to define the actual roles of the Phe431Ser mutation in GyrB and of specific efflux pumps in the resistance phenotype of the *C. freundii* isolate.

The conjugation potential of *qnrB62* was attempted by mating performed between the *C. freundii* isolate and *E. coli* J53, but quinolone resistance was not transferred. Furthermore, pulsed-field gel electrophoresis (PFGE) (14) of the *C. freundii* isolate DNA digested by the enzyme I-CeuI (New England BioLabs) and hybridized to a digoxigenin-labeled *qnrB62* probe (Roche) produced a signal among all the bands detected by a 16S gene probe, revealing that the *qnrB62* gene was chromosomal (Fig. 1a). Most *qnrB*-type genes were located on plasmids (15). However, chromosomal localization of the commonly plasmid-borne *qnrB* genes has been reported in *Citrobacter* isolates (7, 9).

To gain insight into the possible mechanism of *qnrB62* gene dissemination, we investigated its genetic environments. The genetic organization of a complex class 1 integron harboring the *qnrB62* gene was investigated by standard PCR long amplification conditions, implemented with the Expand high-fidelity PCR system (Roche) as per the manufacturer's instructions, followed by DNA sequencing. The specific primers were used as previously described (16). Analysis of a 10,936-bp segment (Fig. 1b) of the *qnrB62* flanking region revealed that *C. freundii* 11-7F456011 harbored a new complex class 1 integron (In1184::IS*CR1*::*qnrB62*, named as previously described [16]). A new class 1 integron (In1184) containing a unique gene array (bla_{VIM-2}-*aacA4′-8-gucD*) was identified upstream of the IS*CR1* element. In comparison with the nucleotide sequences of other class 1 integrons, the *aacA4′-8* gene of In1184 shared 99.8% identity with those of In886 (Korea), In1008 (Poland), In852 (Germany), In38 (Argentina), and In432 (Korea). The bla_{VIM-2} gene of In1184 was present in three out of the five integrons. This result indicates that the unique gene array of In1184 can be generated by cassette transfer from one integron to another or the reassortment of gene cassettes within a particular integron (17).

IS*CR1* may belong to a member of an extended family of IS*91*-like elements that can transpose adjacent DNA sequences by a mechanism termed rolling-circle transposition (and homologous recombination) and may be responsible for the mobilization of class 1 integrons (16–18). A recombination crossover site (RCS) (33-bp DNA sequence containing *ori*IS) at which the insertion of resistance genes into the complex class 1 integron containing IS*CR1* occurs was observed downstream of IS*CR1* (Fig. 1b), as previously described (17). Furthermore, 18 nucleotides at the 3′ end of the *pspA* gene [*pspA*(Δ*18*)] were deleted just downstream of the RCS 3′ end, suggesting a past transposition event mediated by IS*CR1* (Fig. 1b). This is consistent with the finding that the *sapC* gene, just downstream of the RCS 3′ end, was truncated at its 3′ end in In37::IS*CR1*::*qnrB10* and In131::IS*CR1*::*qnrB10* (16). These results indicate that In1184::IS*CR1* can be mobilized next to the gene array [*pspA*(Δ*18*)-*pspF-qnrB62*] commonly associated with *Citrobacter* isolates (*C. freundii* F2503, *C. freundii* S50552, and *C. braakii* ATCC 51113^T in Fig. 1b), as previously described (19). To the best of our knowledge, this is the first report of a complex class 1 integron simultaneously carrying the bla_{VIM-2} and *qnrB* allele in

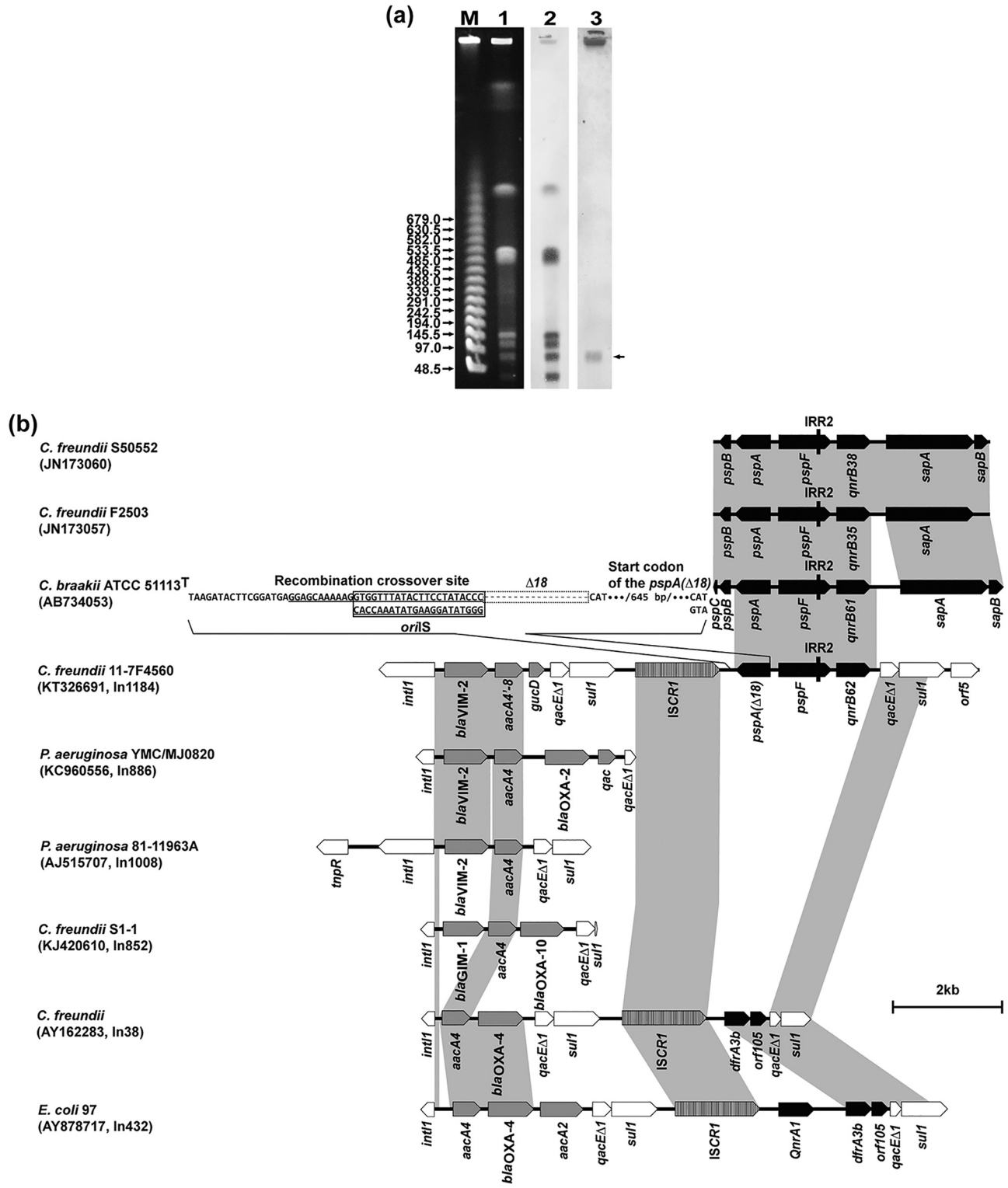

FIG 1 (a) Southern blotting of I-CeuI-digested genomic DNA of *C. freundii* 11-7F4560 carrying the *qnrB62* gene. Lanes: M, lambda ladder PFGE marker (Bio-Rad); 1, I-CeuI restriction banding pattern of genomic DNA from *C. freundii* 11-7F4560 carrying the *qnrB62* gene; 2, Southern blotting of genomic DNA with a probe specific to the 16S rRNA gene; 3, Southern blotting of genomic DNA with a probe specific to the *qnrB62* gene. The band containing the *qnrB62* gene is marked with an arrow. (b) The genetic map of chromosomal DNA from *C. freundii* 11-7F4560 carrying *qnrB62* located a new complex class 1 integron (In1184::IS*CR1*::*qnrB62*), *C. freundii* F2503 carrying *qnrB35*, *C. freundii* S50552 carrying *qnrB38*, *C. braakii* ATCC 51113^{T} carrying *qnrB61* and five different integrons (In886 from *P. aeruginosa* YMC/MJ0820, In1008 from *P. aeruginosa* 81-11963A, In852 from *C. freundii* S1-1, In38 from *C. freundii*, and In432 in plasmid pK097 from *E. coli* 97). Genes are represented by arrow boxes. Genes identified in In1184 are $bla_{\text{VIM-2}}$ (encoding metallo-β-lactamase VIM-2), *aacA4′-8* [encoding aminoglycoside (6)′-*N*-acetyltransferase], *gucD* (open reading frame of a gene of unknown function), *pspA*(Δ*18*) (phage shock protein A gene with an 18-nucleotide 3′ deletion), *pspF* (encoding phage shock protein F), and *qnrB62* (encoding a quinolone resistance determinant). The 5′-CS and 3′-CS are indicated by white arrow boxes. Vertical black bars represent IRR2. The constant (or similar) regions are indicated by gray shading. In an enlargement of the region between the recombination crossover site (RCS) and *pspA*(Δ*18*), the RCS is underlined. *ori*IS (the initiation site of IS*CR1* transposition) is boxed and underlined. Δ*18* (dotted line box) is an 18-nucleotide 3′ deletion (dashed lines) in a 691-bp open reading frame encoding PspA.

a *Citrobacter* clinical isolate resistant to fluoroquinolones and carbapenems.

The mobilization of *qnrB62* and/or regions surrounding *qnrB62* was investigated. An inverted repeat region (IRR [CTGAATTAC TGGGT]) was detected within the coding sequence of the *pspF* gene of In1184::IS*CR1*::*qnrB62*. The IRR is also found at the same position in the chromosome of *Citrobacter* spp. (GenBank accession no. AB734053, AB734055, JN173057, JN173060, AB734055, AB734054, KP339258, KP339259, KP339261, KP339262, and KP339263) and in the plasmids of different *Enterobacteriaceae* species (GenBank accession no. EU523120, JN995611, JX101693, GU295957, JX424423, JX298080, EU643617, and NG_036730). This IRR is similar (0- to 5-bp mismatches) to IRR2, which was previously implicated in the mobilization of other *qnrB* genes to plasmids (19, 20). This mobilization potentiates the dissemination of *qnrB62* in the clinical setting.

Accession number(s). The nucleotide sequences of the *qnrB62* gene and the region surrounding the *qnrB62* gene have been deposited in GenBank under accession no. JX987101 and KT326691. The new complex class 1 integron sequence has been uploaded into the integron database (INTEGRALL; http://integrall.bio.ua.pt/) under integron no. In1184. Therefore, the complex integron was designated In1184::IS*CR1*::*qnrB62*.

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