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ARTICLE

Complex integrons containing *qnrB4-ampC* (bla_{DHA-1}) in plasmids of multidrug-resistant *Citrobacter freundii* from wastewater

Grace Yim, Waldan Kwong, Julian Davies, and Vivian Miao

Abstract: Microbial populations in wastewater treatment plants (WWTPs) are increasingly being recognized as environmental reservoirs of antibiotic resistance genes. PCR amplicons for plasmid-mediated quinolone resistance determinants *qnrA*, *qnrB*, and *qnrS* were recorded in samples from a WWTP in Vancouver, British Columbia. Six strains of ciprofloxacin-resistant *Citrobacter freundii* were isolated and found to carry mutations in *gyrA* and *parC*, as well as multiple plasmid-borne resistance genes, collectively including *qnrB*; *aac(6′)-Ib-cr*; β-lactamase-encoding genes from molecular classes A (bla_{TEM-1}), C (*ampC*), D (bla_{OXA-1}, bla_{OXA-10}); and genes for resistance to 5 other types of antibiotics. In 3 strains, large (>60 kb) plasmids carried *qnrB4* and *ampC* as part of a complex integron in a 14 kb arrangement that has been reported worldwide but, until recently, only among pathogenic strains of *Klebsiella*. Analysis of single-nucleotide polymorphisms in the *qnrB4-ampC* regions infers 2 introductions into the WWTP environment. These results suggest recent passage of plasmid-borne fluoroquinolone and β-lactam resistance genes from pathogens to bacteria that may be indigenous inhabitants of WWTPs, thus contributing to an environmental pool of antibiotic resistance.

Key words: InRBDHA, complex class 1 integron, single-nucleotide polymorphism (SNP), molecular epidemiology.

Résumé : Les populations microbiennes des usines de traitement des eaux usées sont de plus en plus reconnues pour constituer des réservoirs environnementaux de gènes de résistance aux antibiotiques. Des amplicons PCR des déterminants de résistance à la quinolone *qnrA*, *qnrB* et *qnrS* véhiculés par plasmides ont été rapportés dans des échantillons d'une usine de traitement des eaux usées de Vancouver en Colombie-Britannique. Six souches de *Citrobacter freundii* résistantes au ciprofloxacin ont été isolées, et elles se sont avérées comporter des mutations dans les gènes *gyrA* et *parC*, et contenir de nombreux gènes de résistance comprenant *qnrB*, *aac(6′)-Ib-cr*, les gènes codant des β-lactamases des classes moléculaires A (bla_{TEM-1}), C (*ampC*) et D (bla_{OXA-1}, bla_{OXA-10}), ainsi que des gènes de résistance à 5 autres types d'antibiotiques. Chez trois souches, de grands plasmides (>60 kb) comportaient *qnrB4* et *ampC* au sein d'un intégron complexe dans un arrangement de 14 kb qui avait déjà été rapporté à travers le monde, mais qui n'avait été détecté que chez certaines souches pathogènes de *Klebsiella* jusqu'à présent. L'analyse des polymorphismes nucléotidiques des régions *qnrB4-ampC* permet de déduire à 2 introductions dans l'environnement de l'usine de traitement des eaux usées. Ces résultats suggèrent que les gènes de résistance à la fluoroquinolone et aux β-lactames ont été récemment transférés, par l'intermédiaire de plasmides, des pathogènes aux bactéries qui peuvent être des résidants indigènes des usines de traitement des eaux usées, contribuant ainsi à un pool environnemental de résistance aux antibiotiques. [Traduit par la Rédaction]

Mots-clés : InRBDHA, intégron complexe de classe 1, SNP, épidémiologie moléculaire.

Introduction

Antibiotic resistance has been primarily considered a clinical phenomenon, but the movement of resistance genes and host bacteria from human communities into the environment has major general epidemiological consequences for dissemination and persistence (Hawkey and Jones 2009; Keen and Montfort 2012; Martinez et al. 2009). Wastewater treatment plants (WWTPs) are at the interface between human-influenced and environmental compartments, collecting influent from a variety of sources (e.g., domestic, institutional, industrial, agricultural) and processing it to produce an effluent that can be discharged into natural waterways. The quality of the effluent — assessed in terms of reduction of organic matter and biochemical oxygen demand — is dependent on the level of treatment: primary treatment relies on physical processes such as aeration and sedimentation, while secondary treatment also involves additional managed microbial processes (Gomes 2009). WWTPs have diverse and dynamic microbial communities (Figuerola and Erijman 2007) that are exposed to and affected by the collective microbiological and chemical inputs from the catchment area. A variety of antibiotic resistance genes, as well as genetic components that facilitate their dissemination, e.g., integrons (Cambray et al. 2010) and plasmids (Schluter et al. 2007), have been observed in wastewater (Baquero et al. 2008; LaPara and Burch 2012; Tennstedt et al. 2003; Xu et al. 2007). The constant inflow of new material, rich organic conditions, and high microbial density make WWTPs potential "hotspots" for lateral gene transfer and bacterial evolution (Szczepanowski et al. 2009) and a point of access to investigate the overall ecology of antibiotic resistance.

The fluoroquinolones (FQs) are one class of antibiotics for which resistance reservoirs associated with WWTP microbiota may be a concern (Bonemann et al. 2006; Cummings et al. 2011; Koczura et al. 2012). Resistance is primarily chromosomally encoded, and includes nonspecific efflux pumps as well specific mutations in the target proteins, DNA gyrase, and topoisomerase (encoded by the genes *gyr* and *par*), but plasmid-mediated quino-

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lone resistance (PMQR) conferred by the Qnr family of pentapeptide repeat proteins is prevalent (Rodriguez-Martinez et al. 2011; Strahilevitz et al. 2009). The *qnrA*, *qnrB*, and *qnrS* genes are distributed worldwide, while *qnrC* and *qnrD* are more recently described from China and Nigeria, respectively; the *qnrB* group is the most diverse, with over 70 variants (http://www.lahey.org/qnrStudies/). In addition to *qnr*, 2 genes for efflux pumps, *oqxAB* (Zhao et al. 2010) and *qepA* (Baudry et al. 2009), and one for a FQ-modifying enzyme, *aac(6′)-1b-cr*, have been recognized as contributors to PMQR (Rodriguez-Martinez et al. 2011). Most investigations of PMQR have focused on clinical isolates, but evidence exists for an environmental origin of *qnr* genes, with *Shewanella* and *Vibrio* spp. proposed as reservoirs and sources for *qnrA* and *qnrS*, respectively (Cattoir et al. 2007; Poirel et al. 2005). The discovery of *qnrS* in *Aeromonas* spp. further highlights the potential for dissemination through water-borne bacteria (Ishida et al. 2010; Picao et al. 2008).

Usage of FQs has been associated with a rise of resistant pathogens in Canada (Adam et al. 2009), and *qnr* has been observed in the Calgary Health Region (Pitout et al. 2008; Poirel et al. 2006). In British Columbia, β-lactams and FQs are the 1st and 5th, resepctively, most prescribed classes of antibiotics (Li et al. 2010), and surveillance efforts are needed to determine the range and prevalence of PMQR determinants. In this study, samples from a WWTP in the Vancouver area were examined for PMQR genes, and the presence of *qnr* genes is reported.

Materials and methods

Screening for *qnr* and integrons in mixed cultures

One grab sample of wastewater from each of the influent, aeration, post-aeration, and effluent stages was collected in June 2007 from Iona Island WWTP, a primary treatment facility servicing an estimated 600 000 people in Vancouver, British Columbia. Bacterial cells were collected by filtering 300 mL of each sample through 0.22 μm membranes (Millipore Corp.). The cells were resuspended in 5 mL of sterile water, and 50 μL was transferred to 5 mL of Luria-Bertani (LB) broth and incubated at 30 °C overnight. A portion of the overnight cultures was stored in 15% glycerol ("cell stocks") at –80 °C. A crude lysate representing total DNA was prepared from the remainder by alternately freezing (–80 °C, 10 min) and heating (85 °C, 1 min) cells 5 times and collecting the aqueous fraction afterwards for use in the detection of *qnrA*, *qnrB*, and *qnrS* by PCR using specific primers (Kehrenberg et al. 2006). *Escherichia coli* J53 carrying pMG252, pMG298, or pMG308 (Gay et al. 2006; Jacoby et al. 2006; Martinez-Martinez et al. 1998), carrying *qnrA1*, *qnrB1*, or *qnrS1*, respectively, were used as positive controls, and amplicons were sequenced and compared with accessions in NCBI databases using the BLAST programs.

Isolation and characterization of ciprofloxacin-resistant (Cip^R) strains

Strains were isolated by plating –80 °C cell stocks on LB agar supplemented with 5 μg/mL ciprofloxacin. Colonies that appeared after incubation at 30 °C overnight were screened for *qnr* genes by PCR as above, and unique *qnr*-bearing, cip^R strains were identified by 16S rRNA gene sequences and by PCR screening for genes related to integrons, e.g., *intI1*, *intI2*, *intI3*, and gene cassettes, using primers and methods previously described (Xu et al. 2007, 2011). Primer pair FQ1 (Jacoby et al. 2006)/K426 (5′-CTARCCMATVAYMGCRATRCCAA-3′) was used to generate larger *qnrB* amplicons as needed. DNA flanking *qnrB* in Iona 5 was amplified with primers K417 (5′-CTCCGCTGTATGTCACTTTAGC-3′) and K418 (5′-GACACGGGACCAGCGATTACC-3′), designed originally for primer walking pCFI-1 (below). The strains were also tested by PCR with primers published for other genes associated with resistance to quinolones: *gyrA* (Vila et al. 1995), *aac(6′)-Ib* (Park et al. 2006), EC-PARC-A/EC-PARC-B for *parC* (Nishino et al. 1997), and QEPA-F/QEPA-R for *qepA* (Baudry et al. 2009). Plasmid pSTVqepA (Yamane et al. 2008) was used as a positive control for *qepA* PCR. Antibiotic susceptibilities of the strains were determined in duplicate by disk diffusion tests on Müeller-Hinton agar (see Table 1). Commercial antibiotic disks were used (Difco or Oxoid), except for rifampicin, which was prepared by aliquoting 10 μL of 10 mg/mL rifampicin (Sigma) solution onto 6 mm diameter paper disks (AMD Manufacturing, Mississauga, Ontario).

Characterization of plasmids

Plasmids isolated from *Citrobacter freundii* by alkaline lysis were analysed by Southern analysis using a *qnrB4* probe (PCR DIG-Labeling Mix, Roche Applied Science, Germany) amplified with primers qnrB-fw/qnrB-rv (Kehrenberg et al. 2006) and pCFI-2 as a template. Probe hybridization was visualized according to the manufacturer's instructions by chromogenic detection. Plasmid preparations were also electroporated into *E. coli* DH10B followed by selection of transformants on LB agar supplemented with tetracycline 30 μg/mL, trimethoprim 15 μg/mL, or ampicillin 100 μg/mL; these antibiotics were used because the genes for these resistances are linked to *qnrB4* (see results), and provided a robust level of selection. Transformants were profiled for antibiotic susceptibility as above, and their plasmids were isolated for use as templates in PCR to monitor the presence of *qnr*, *int*, and other genes, and to generate large overlapping amplicons for sequencing. Plasmid sizes were estimated with respect to known references, e.g., supercoiled ladder (Invitrogen), and by restriction digestion with *Eco*RI, *Bam*HI, and *Hin*dIII (New England Biolabs).

All sequencing was conducted directly on PCR amplicons (unless otherwise specified) by Macrogen (Korea) or Eurofins MWG Operon (USA), and results were manually assembled using Vector NTI (Invitrogen) and MacVector 12.0.2 (MacVector, Inc.). Five *Eco*RI fragments of pCFI-1 were subcloned into pGEM-T (Promega) and sequenced completely; some regions not subcloned were sequenced by primer walking of pCFI-1. Sequencing from pCFI-2 and pCFI-3 was based on overlapping fragments obtained by PCR from isolated plasmids and was guided by possible similarities to pCFI-1. Sequences for the integrons in pCFI-1, pCFI-2, and pCFI-3 were deposited as GenBank accessions JN215523, JN215524, and JQ356870, respectively; the *qnrB12* region in Iona 5 was deposited as JQ356871.

Results and discussion

Identification of *qnr* in Iona Island WWTP

The importance of WWTPs as repositories of antibiotic resistance has been noted (Tennstedt et al. 2003; Zhang et al. 2009; Schluter et al. 2007), and there is increasing evidence for PMQR genes in these environments. Amplicons indicative of *qnr* genes were detected in DNA from each of the 4 stages sampled (influent, aeration, post-aeration, and effluent). The sequence of PCR products generated from the influent and effluent using *qnrA*-specific primers were identical to that of *qnrA1* (570/570 nucleotides, GenBank accession AY070235); similarly, the sequence of an amplicon obtained from the influent using *qnrS*-specific primers was identical to that of *qnrS1* (524/524 nucleotides, GenBank AB187515). The presence of mixed base positions in amplicons obtained with *qnrB*-specific primers suggested the possibility of multiple *qnrB* genes, and this was confirmed by studies of isolated strains (below). These results indicate that the 3 main classes of *qnr* genes are present in the Vancouver region.

Ciprofloxacin-resistant (Cip^R) isolates

Cultivable bacteria carrying *qnr* genes were recovered from the wastewater samples for more detailed analysis. Twenty-one of 565 strains isolated on agar containing 5 μg/mL ciprofloxacin were found to carry *qnrB* by PCR screening; no *qnrA*- or *qnrS*-bearing strains were recovered. The 21 strains were characterized by antibiotic susceptibility and other genetic tests, and 6 unique strains were identified: 4 (designated Iona 1 to Iona 4) from the

Table 1. Selected characters of *Citrobacter freundii* strains and *Escherichia coli* transformants.

Strain	*qnr*	*aac(6′)-Ib-cr*	*intI*	Integron cassettes	Resistance to antibiotics[a]		
					Quinolone	β-Lactam	Other
C. freundii							
Iona 1	*B6*∼	+	–	–	Cip, Nal	Amp, Cfm, Cpd, Fox, Kz	Kan
Iona 2 (pCFI-1)	*B6*∼, *B4*	+	*intI1*	*aac(6′)-Ib-cr*, bla_{OXA-1}, *catB3, arr3*	Cip, Nal	Amp, Caz*, Cfm, Cpd, Cro*, Ctt, Fox, Kz, Prl, Tzp*	Kan, Rif, SxT, Tet, Tmp
Iona 3	*B6*∼	+	–	–	Cip, Nal	Amp, Cfm*, Cpd, Fox, Kz, Prl	Kan, SxT, Tet[b], Tmp
Iona 4 (pCFI-2)	*B4*	–	*intI1*	*dfrA14, arr2, cmlA5,* bla_{OXA-10}*, aadA1*	Cip, Nal	Amp, Caz*, Cfm, Cpd, Cro*, Ctt, Fox, Kz, Prl	Cml, Kan, Rif, Str, Spc, SxT, Tmp
Iona 5	*B12*	–	–	–	Cip, Nal	Amp, Cfm, Cpd, Fox, Kz, Prl*, Tzp*	
Iona 6 (pCFI-3) (pCFI-4)	*B4*	+	*intI1*	*aac(6′)-Ib-cr*, bla_{OXA-1}, *catB3, arr3*	Cip, Nal	Amp, Caz, Cfm, Cpd, Ctt*, Cro*, Fox, Kz, Prl, Tzp*	Kan*, Rif, Spc*, SxT, Tet, Tmp
			intI1	*dhfrA1, aadA1*			
E. coli							
E. coli::pCFI-1	*B4*	+	*intI1*	*aac(6′)-Ib-cr*, bla_{OXA-1}, *catB3, arr3*		Amp, Cfm, Cpd, Kz, Prl	Kan*, Rif, SxT, Tet, Tmp
E. coli::pCFI-2	*B4*	–	*intI1*	*dfrA14, arr2, cmlA5,* bla_{OXA-10}*, aadA1*		Amp, Cfm, Cpd, Kz	Cml, Kan, Rif, Spc*, SxT, Tmp
E. coli::pCFI-3	*B4*	+	*intI1*	*aac(6′)-Ib-cr*, bla_{OXA-1}, *catB3, arr3*		Amp, Cfm, Cpd, Fox*, Kz, Prl*, Tzp*	Kan*, Rif
E. coli::pCFI-4	–	–	*intI1*	*dfrA1, aadA1*		Cfm*	Spc*, SxT, Tet, Tmp

[a]Antibiotics tested: ampicillin (Amp), 10 μg; cefixime (Cfm), 5 μg; cefotetan (Ctt), 30 μg; cefoxitin (Fox), 30 μg; cefpodoxime (Cpd), 10 μg; ceftazidime (Caz), 30 μg; ceftriaxone (Cro), 30 μg; cephazolin (Kz), 30 μg; chloramphenicol (Cml), 30 μg; ciprofloxacin (Cip), 5 μg; kanamycin (Kan), 30 μg; nalidixic acid (Nal), 30 μg; piperacillin (Prl), 100 μg; piperacillin/tazobactam (Tzp), 100 μg/10 μg; rifampicin (Rif), 100 μg; spectinomycin (Spc), 100 μg; streptomycin (Str), 10 μg; sulfimethoxazole/trimethoprim (SxT), 25 μg; tetracycline (Tet), 30 μg; trimethoprim (Tmp), 5 μg. An asterisk (*) indicates intermediate susceptibility. CLSI breakpoints for *Enterobacteriaceae* (Clinical and Laboratory Standards Institute 2011) were used as a guide for most assessments of susceptibility. Strains with Spc-associated inhibition zones <11 mm were designated resistant; 12–18 mm, intermediate; and >19 mm, sensitive. The *rpsL* gene in the *E. coli* (DH10B) host confers resistance to Str in transformants (not shown). Resistance to levofloxacin (5 μg), imipenem (10 μg), and meropenem (10 μg) was not observed among Iona strains and was not tested in the transformants.

[b]A *tetA(D)* gene was detected by PCR screening with primers described in Xu et al. (2011) in Iona 3.

influent, and 2 (Iona 5 and Iona 6) from the effluent (Table 1). All 6 were recognized as *C. freundii* by their 16S rRNA gene sequences (1322 nucleotides, >99% identity to *C. freundii* ATCC 8090, GenBank AJ233408.1). Sequences of *gyrA* and *parC* amplicons (below) were also consistent with *C. freundii* (e.g., 99% and 98% identity with GenBank accession Nos. AF052253 and AF056286, respectively). This result was unexpected, as *Escherichia* and *Klebsiella* spp. are also abundant in wastewater and, in some cases, known to be FQ resistant (Mokracka et al. 2011). While a preponderance of *qnrB* alleles has been notably associated with *Citrobacter* (Jacoby et al. 2011), larger studies with additional isolation procedures and higher throughput detection methods are needed to explore this observation and its potential significance, and to extend the search for organisms carrying *qnrA1* and *qnrS1*.

Characterization of *qnrB*-carrying *Citrobacter* strains

In addition to resistance or partial resistance to representative quinolones (nalidixic acid, ciprofloxacin, and levofloxacin), resistance to a number of β-lactams and at least one other class of antibiotic (Table 1) was observed. Resistance to the β-lactams ampicillin, cephazolin, cefoxitin, cefpodoxime, and cefixime was exhibited by all strains, likely reflecting contributions from both chromosomal and plasmid-borne resistance determinants (Barlow and Hall 2002). Iona 2, Iona 4, and Iona 6 were resistant or partially resistant to additional β-lactams (cefotetan, ceftriaxone, and ceftazidime), but all 6 strains were susceptible to imipenem and meropenem. Increased susceptibility to piperacillin in the presence of the inhibitor tazobactam suggested the presence of molecular class A (Bush and Jacoby 2010) β-lactamases (e.g., TEM-1) in Iona 2, Iona 3, Iona 4, and Iona 6. Notably, Iona 6, with resistance or partially reduced susceptibility to antibiotics of 5 other classes, was recovered from the WWTP effluent.

Genetic screening by PCR of the isolated strains revealed the presence of mutations known to reduce FQ susceptibility (Nishino et al. 1997): *gyrA* encoded a T83I substitution in DNA gyrase in all strains, and *parC* encoded a S80I substitution in topoisomerase II in all strains but Iona 6. Four strains carried *aac(6′)-Ib-cr*, but none carried *qepA*. Strains were also tested for the presence of integrons, as these are associated with antibiotic resistance and have been found in WWTP (Tennstedt et al. 2003). PCR assays showed the presence of an integrase gene (*intI1*), gene cassettes, and an ISCR1 (*orf513*) element indicative of a complex class 1 integron (Toleman et al. 2006) in Iona 2, Iona 4, and Iona 6 (details below). No evidence for class 2 or class 3 integrons was detected, although interestingly, class 3 integrons have been recovered in another regional WWTP in southern British Columbia (Xu et al. 2007).

qnrB genes in isolated strains

Sequencing of *qnrB* amplicons (corresponding to amino acids 48–200) (Jacoby et al. 2008) obtained with the screening primers from the *C. freundii* strains indicated the presence of different *qnrB* genes, and additional characterization was conducted. In Iona 1 and Iona 3, analysis of a longer PCR product suggested the presence of a *qnrB6*-like gene (i.e., *qnrB6* or *qnrB9*, or a novel allele including the mutations characteristic of either), but this could not be further resolved as the distinguishing residue (Ile or Val) was in the primer-binding site. Analysis of a 1.1 kb amplicon showed that Iona 5 carried *qnrB12* (99.4% DNA, 100% amino acid identity to GenBank AM774474.1, CA082104, respectively); the short stretches (135 and 295 bases) flanking it were also highly similar (97.8% and 98.2% identity, respectively) to tracts adjacent to the known *qnrB12* gene. As part of the repertory of PMQR, *qnr* genes are typically considered plasmid-encoded; however, it has been recently suggested that most *qnrB* genes are likely to be chromosomal, with only a few alleles, such as *qnrB4*, having been mobilized (Jacoby et al. 2011). Studies have placed *qnrB6* and *qnrB12* in the chromosome of *Citrobacter* spp., for example (Sanchez-Cespedes et al. 2009; Kehrenberg et al. 2008). Iona 6 not only carried a *qnrB6* gene, identified in the initial PCR screen, but also a complete *qnrB4* gene. The latter (identical to GenBank DQ303921) was also found in Iona 2 and Iona 4, and *E. coli* transformation, southern analyses, and sequencing were used to char-

acterize the pCFI (for *Citrobacter freundii* Iona) plasmids bearing *qnrB4* genes in these 3 strains.

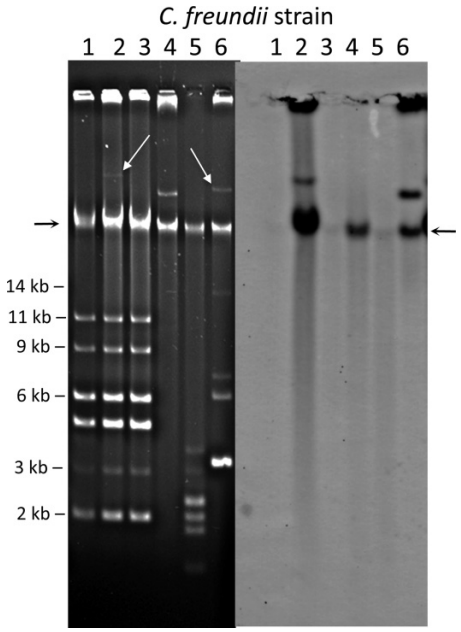
Genetic context of *qnrB4* in plasmids

Plasmid pCFI-1 (~80 kb) was observed as a *qnrB4*-hybridizing band in southern analysis of plasmids from Iona 2 (Fig. 1). Three regions totaling 69 kb were sequenced (Figs. 2A–2C), and the *intI1* and *qnrB4* genes were revealed as part of a complex class 1 integron comprising most of a 23.6 kb contig (Fig. 2C). This integron was essentially identical (12 point mutations and 2 indels in 22 014 nucleotides) to InRBDHA in pRBDHA, a plasmid from a multidrug-resistant clinical isolate of *Klebsiella pneumoniae* (Verdet et al. 2006). The variable vr-1 region contains 4 gene cassettes, *aac(6′)-Ib-cr*, bla_{OXA-1}, *catB3*, and *arr-3*, associated with resistance to FQ and aminoglycosides, ampicillin, chloramphenicol, and rifampicin, respectively. Iona 2 was chloramphenicol sensitive, however, suggesting that *catB3* expression is below detection (Picao et al. 2008). (This vr-1 array carries a short proximal duplication (Quiroga et al. 2007) of otherwise distal 3′ conserved sequences that may lead to amplification of a PCR product diagnostic for a "cassette-less" integron in surveys, and is mentioned here as a cautionary note in interpretation of PCR screening results). The vr-2 region of the complex integron is composed of *qnrB4*, flanked upstream by genes for hypothetical proteins (*sap* region) and downstream by genes for phage shock proteins (*psp* region) and *ampC* (bla_{DHA-1}) *ampR*. Elsewhere on pCFI-1, 4 resistance genes, bla_{TEM-1}, *dfrA26*, *sul2*, and *tetA(D)*, as well as an arsenic resistance operon (*arsRDABC*) were observed amidst genes for plasmid maintenance and transposases (Figs. 2A and 2B). These genes account for much of the phenotype of Iona 2 (e.g., increased susceptibility to piperacillin in the presence of tazobactam is consistent with the presence of bla_{TEM-1}), as well as the spectrum of acquired resistances in the corresponding *E. coli* transformant (Table 1). The presence of *qnrB* and the *aac(6′)-Ib-cr* cassette did not lead to a Cip^R phenotype in the *E. coli* transformant, indicating that reduced susceptibility to ciprofloxacin in Iona 2 was primarily due to mutations in *gyrA* and *parC*.

A low copy plasmid, pCFI-2 (~75 kb) was difficult to obtain in quantity from the source isolate, Iona 4, but was isolated after transformation of *E. coli*. Sequencing of a 24.5 kb region showed the *intI1* and *qnrB4* fragments originally detected by PCR to be part of a complex integron (Fig. 2D), with a 5-cassette vr-1 array carrying *dfrA14*, *arr2*, *cmlA5*, bla_{OXA-10}, and *aadA1* that accounts for the observed resistance to trimethoprim, rifampicin, chloramphenicol, some β-lactams, and kanamycin, respectively, in Iona 4 and its corresponding *E. coli* transformant. This array resembled InRDDHA from a clinical strain of *K. pneumoniae* (Verdet et al. 2006), but the adjoining vr-2 region (with *qnrB4*) was identical to that of pCFI-1 at all but 3 nucleotides.

Mapping regions around *intI* and *qnrB4* in the ~60 kb Iona 6 plasmid pCFI-3 (Fig. 1, right panel) suggested the presence of yet another InRBDHA-like element. This was confirmed by sequencing a 22 634 bp region that exhibited only 2 nucleotide differences from the equivalent region of pCFI-1. Other regions observed in pCFI-1, however, such as the arsenic operon, *tetD*, Tn*3*-bla_{TEM}, and the ISCR2 to *dfrA* region (Figs. 2A and 2B) were not detected. Phenotypes associated with antibiotic resistance genes in the complex integron were observed in an *E. coli* transformant carrying pCFI-3, but other resistances, e.g., tetracycline, expressed by Iona 6 were conferred by genes carried by pCFI-4 (~27 kb), another plasmid that was recovered by transformation. A class 1 integron in pCFI-4 bearing *dfrA1* and *aadA1* gene cassettes encoding resistance to trimethoprim and spectinomycin or streptomycin explained phenotypes observed in Iona 6 and the *E. coli* transformant. This array is also present in other enterics such as *E. coli* (GenBank CP001856).

Fig. 1. Plasmids in *Citrobacter freundii*. (Left panel) Ethidium-stained plasmids (sizes of some representatives are shown) in 1% agarose gel after electrophoresis in TAE at 70 V for 3 h. White arrows mark positions of faintly staining high molecular mass plasmids. (Right panel) Blot of gel probed with *qnrB4*. Hybridization to chromosomal DNA (black arrow) is likely due to damaged (linearized) plasmids co-migrating with contaminating host DNA, but the possibility of integrated forms of the plasmids cannot be excluded.



Comparison of *qnrB4 and ampC* (bla_{DHA-1}) regions from different sources

An association between transmissible *qnrB4* genes with *ampC* (bla_{DHA-1}) has been noted in *Enterobacteriaceae* (Strahilevitz et al. 2009), particularly among clinical strains of *Klebsiella*. The ~14 kb vr-2 regions containing *qnrB4* and *ampC* (bla_{DHA-1}) in the Iona strains were nearly identical to each other and InRBDHA, as well as to elements in strains reported from a variety of geographical sources (Fig. 3). Most were found in complex integrons or associated with transposase genes, and nearly all were noted or inferred to be plasmid-borne. It has been proposed, following detailed analysis of *Klebsiella* plasmid pRBDHA (Verdet et al. 2006), that an ancestral form of integron InRBDHA acquired *ampC* (bla_{DHA-1}) from the chromosome of *Morganella morganii*, then later added the *sap-qnrB4-psp* region via ISCR1-mediated transposition. The *sap-qnrB4-psp* region is suggested to derive from the chromosome of *Citrobacter youngae* (Jacoby et al. 2011), a member of the *C. freundii* complex. Interestingly, the *ampC* (bla_{DHA-1}) region, suggested as the earlier acquisition, appears more similar to *Morganella* chromosomal DNA (e.g., 98.4% identity to accession AF055067) than the *sap-qnrB4-psp* region is to *C. youngae* chromosomal DNA (the latter at 90.2% identity, notwithstanding a 1 kb indel as noted by Jacoby et al. (2011)); however, this might be due to greater functional constraints of the former, or there may be additional intermediates in the provenance of this integron segment. While it is possible that *qnrB* was part of the *Citrobacter* genome ancestrally, given a complex multistage origin versus the high similarity to other contemporary plasmid-borne elements, it is more probable that the *qnrB4-ampC* (bla_{DHA-1}) regions in Iona 2, Iona 4, and Iona 6, which represent the first examples of this vr-2 region to be completely described from *C. freundii*, were acquired "ready-made" horizontally from an pRBDHA-like plasmid.

Fig. 2. Maps of antibiotic resistance regions in pCFI-1, pCFI-2, and pCFI-3. (A) ISCR2 and associated resistance genes from pCFI-1. (B) Arsenic resistance operon *arsRDABC* from pCFI-1. (C) Complex integrons from pCFI-1 and pCFI-3. (D) Complex integron from pCFI-2. (E) Double disk synergy tests on Müeller–Hinton agar using the inducer-substrate combination cefoxitin (x) – ceftazidime (z) on *Citrobacter freundii* Iona 1 through Iona 6 (panels i–vi). Genes are represented by block arrows: dark and light gray arrows indicate resistance genes or integron associated elements, respectively. Locations of variable regions (vr-1, vr-2) typical of complex integrons are indicated. In C and D, features include conserved integron genes (light grey), cassette resistance genes (dark gray), *qnrB4*(black) and flanking regions (diagonal stripes) typically linked with *qnrB4*, and *ampR* and *ampC* (bla_{DHA-1}) (vertical stripes). The region of near-identity in C and D is boxed with a broken line.

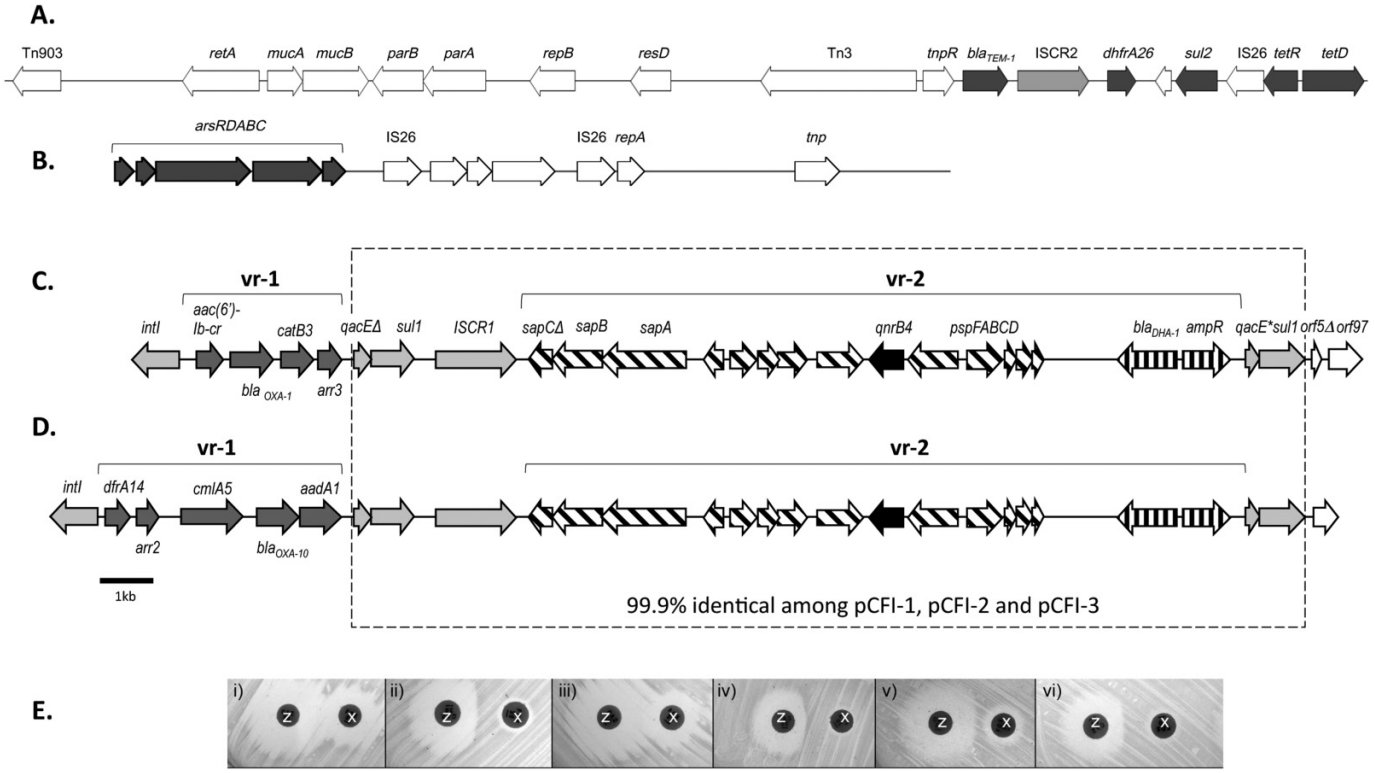


Fig. 3. Comparison of *qnrB4* flanking regions relative to pCFI-1. The 13 986 bp vr-2 region of pCFI-1 (nt 7368–21353) is used as a reference (top). Solid and dashed lines represent regions of >99% or ~90% identity, respectively, to sequences from other bacteria (Cfr, *Citrobacter freundii*; Cyo, *Citrobacter youngae*; Eco, *Escherichia coli*; Kox, *Klebsiella oxytoca*; Kpn, *Klebsiella pneumoniae*) and geographic origin; tick marks represent position of bases that differ from pCFI-1. GenBank accession number (in parentheses) and sequence designation at right. In some sequences, ISCR1 is replaced by *tnpA* (IS26).

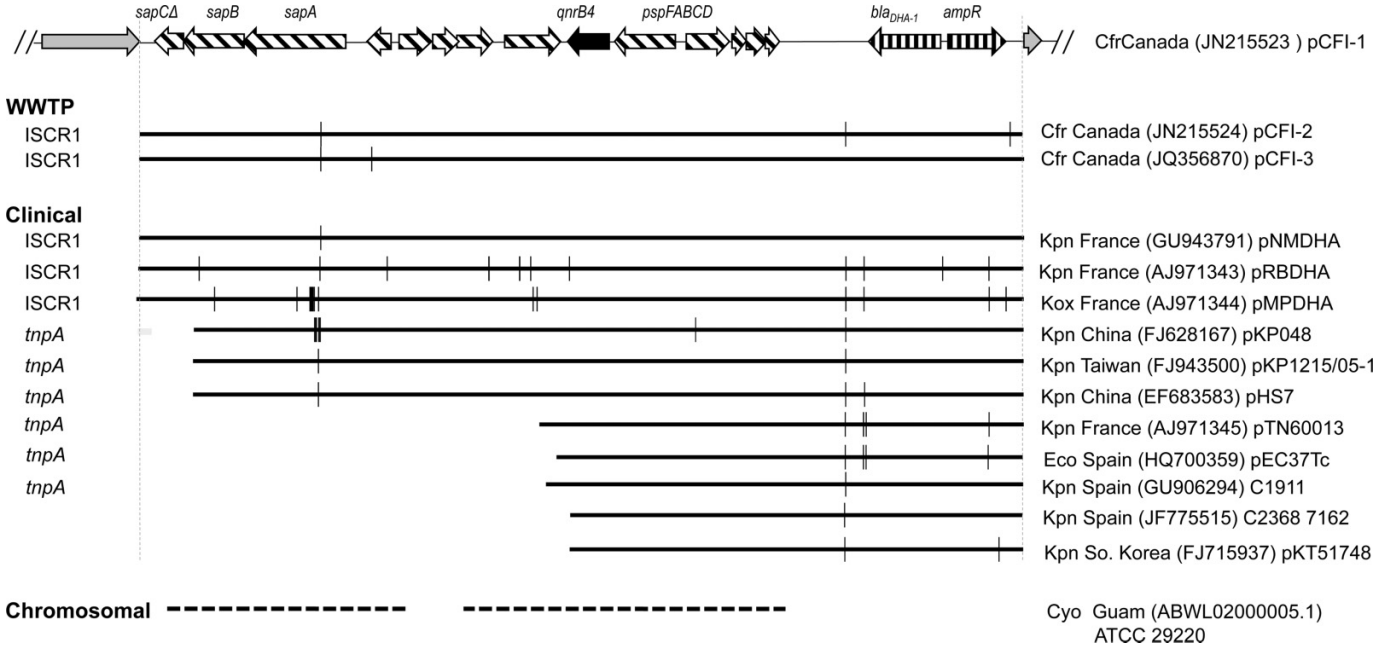
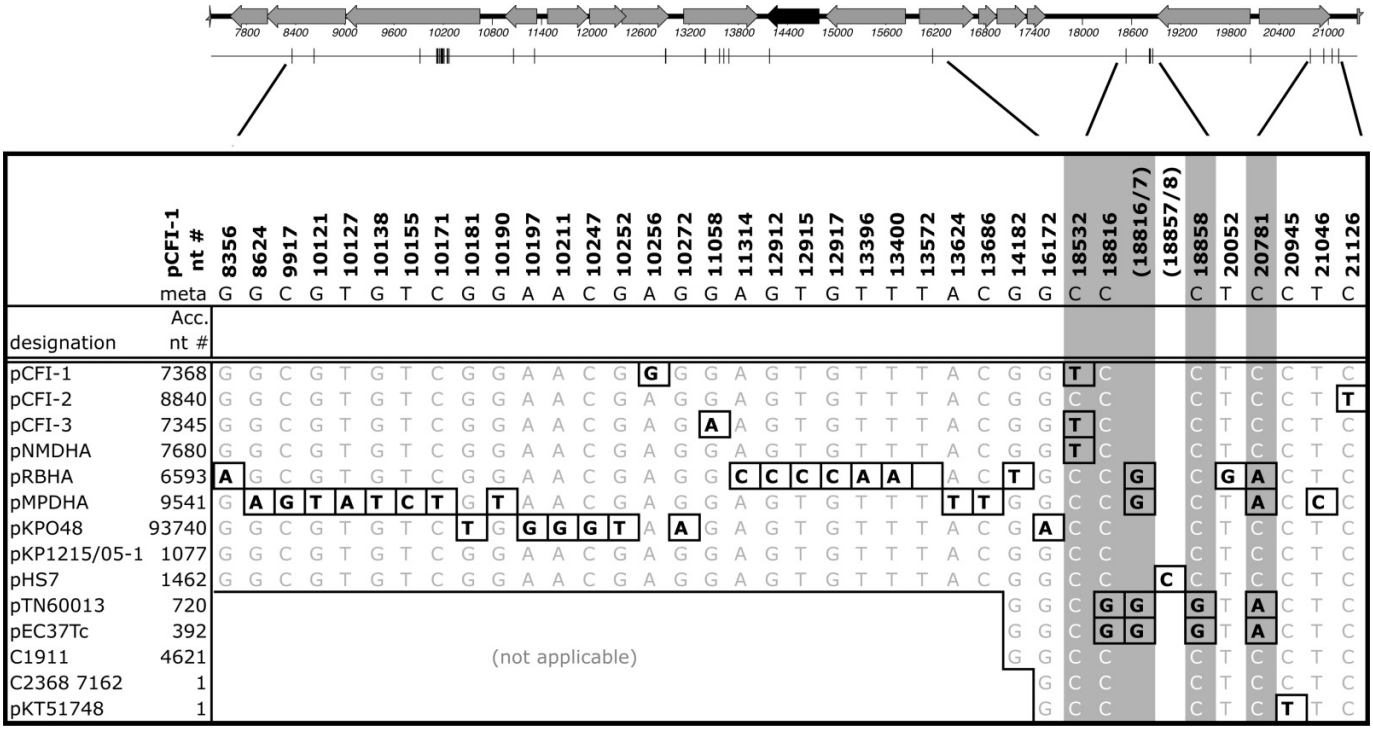


Fig. 4. Single-nucleotide polymorphism in *qnrB4-ampC* (bla_{DHA-1}) region relative to a "meta" reference sequence. Only bases at variable positions in the vr-2 region (see Fig. 3) are shown. The native nucleotide number at the start of the sequenced region used for comparison in each accession is indicated ("Acc. nt#"). The variable positions are identified by the pCFI-1 equivalent location ("pCFI-1 nt#"); tick marks in the reference map indicate where these positions are.



Utility of single-nucleotide polymorphisms in marking plasmid components

In addition to exploring larger questions such as the evolutionary progenitors of particular genes (above), detailed examination of highly similar, laterally-transferred regions of plasmids may also reveal features such as indels and single-nucleotide polymorphisms (SNPs) that reflect the recent history of specific components. Comparison of the most similar *qnrB4* and *ampC* (bla_{DHA-1}) regions (Fig. 3) against a consensus or "meta" reference sequence constructed from the majority base call at each variant position revealed 38 differences, with the greatest number of unique changes occurring in integrons from *K. pneumoniae* isolates from China and France (pKP048, pRBDHA, pMPDHA). Differences at 5 positions at the 3′ end (of which 4 were in presumptive noncoding regions between *pspD* and *ampC* (bla_{DHA-1}) or *ampR*) were shared by at least 2 elements (Fig. 4). Insertion of a G after position 18816 (pCFI-1 nucleotide numbering) distinguishes the *qnrB4-ampC* (bla_{DHA-1}) regions in 3 *Klebsiella* strains reported in France from 2 others in Spain. Interestingly, the sole *E. coli* representative (from Spain) also carried the G insertion, suggesting that its vr-2 region may have a closer historical affinity to those in *Klebsiella* from France, than to 2 populations from Spain. The near identity of the vr-2 regions of the pCFI plasmids — also most similar to that in another plasmid from France, pNMDHA — indicates a recent introduction into the Iona WWTP catchment. However, a C/T SNP at position 18532 is shared by pCFI-1, pCFI-3, and pNMDHA, but not by pCFI-2, and notably, the last also has a different vr-1 region. This suggests the possibility of 2 independent introductions of the *qnrB4-ampC* (bla_{DHA-1}) region into *Citrobacter*, either already associated with different vr-1 regions, or acquiring it within the WWTP. Thus, even though overall plasmid architectures are susceptible to rearrangement by recombination and transposition, examination of shared SNPs or other local characters in elements from different sources may be informative epidemiologically if there are a sufficient number of contemporary representatives.

Conclusions

Together with detection of *qnrB* at a pilot WWTP on the University of British Columbia campus, and *qnrB* and *qnrS* at another regional WWTP (W. Kwong and J. Davies, unpublished), the results of this study indicate that various *qnr* genes may have been broadly distributed in the Vancouver area for some time, at least since 2007. The finding in wastewater of multidrug-resistant *C. freundii* with plasmids containing *qnrB4* regions characteristic of clinical strains of *Klebsiella* supported the concept of an environmental reservoir of resistance. Development of specific qPCR probes for *qnr* and linked resistance gene will be needed to further investigate the prevalence of environmental PMQR; of particular importance will be its relation to usage levels of FQs in the corresponding area. In some cases, exploration of molecular genetic features such as SNPs may also facilitate epidemiological monitoring and source tracking of resistance determinants. Altogether, investigation of WWTP microbiota and their environments will continue to enhance our understanding of the ecological cycle of antibiotic resistance, and may enable practical interventions to reduce the traffic of resistance genes in the future.

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