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Citrobacter spp. as a source of *qnrB* alleles

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ABSTRACT

qnrB is the most common of the five *qnr* families and has the greatest number of allelic variants. Almost two thirds of the *qnrB* alleles have been reported in *Citrobacter* spp., and several were shown to be located on the chromosome. In this study PCR was used to investigate the prevalence of plasmid-mediated quinolone resistance genes in 71 clinical isolates belonging to the *Citrobacter freundii* complex. Thirty-seven percent contained *qnrB* alleles, including 7 (*qnrB32 to qnrB38*) that were novel and one pseudogene, while none contained *qnrA, qnrC, qnrD, qnrS,* or *aac(6')-Ib-cr*. When the strains were arrayed by related 16S rRNA sequence and further separated into subspecies by biochemical criteria, clustering of *qnrB* positive strains was evident. In only two strains with *qnrB2* and *qnrB4* was quinolone resistance transferable by conjugation, and only these strains contained the IS*CR1* sequence that is often associated with *qnrB* on plasmids. Five of 26 *qnrB* positive strains contained integrase genes, but these included the strains with *qnrB2* and *qnrB4* as well as two strains with other transmissible plasmids. In a fully sequenced genome of *Citrobacter youngae*, a member of the *C. freundii* complex, another novel *qnrB* allele, *qnrB39,* occurs in a sequence of genes that is 90% identical to sequence surrounding integron-associated *qnrB4* incorporated into plasmids. The chromosome of *Citrobacter* is the likely source of plasmid-mediated *qnrB*.

INTRODUCTION

Of the five known *qnr* families *qnrB* is the most common worldwide and has the greatest number of alleles (21). Curiously, almost two thirds of the *qnrB* alleles were discovered in isolates of the genus *Citrobacter*, which also contains *qnrB* positive isolates from the pre-antibiotic era (18). *qnrA* and *qnrS* have likely origins in chromosomal genes from *Shewanella algae* (16) and *Vibrio splendidus* (2), respectively, but the origin of *qnrB* is not known. The aim of this study was to determine the prevalence, variety, and location of *qnrB* genes in a contemporary sample of *Citrobacter* hospital isolates and to investigate *Citrobacter* spp. as the source of *qnrB*. .

MATERIALS AND METHODS

Bacterial strains and plasmids. Consecutive strains (one per patient) identified as *Citrobacter freundii* in the clinical microbiology laboratories at the Lahey Clinic or the Massachusetts General Hospital (MGH) were collected between September, 2009 and May, 2010. Plasmid pBC SK (*cat*) (Agilent Technologies, Santa Clara, CA) was used for cloning.

Susceptibility testing. Antibiotic susceptibility as evaluated by disk diffusion on Mueller-Hinton agar (Becton, Dickinson, and Co., Sparks, MD) following CLSI criteria (4). Ciprofloxacin MICs were determined with the same media by Etest (bioMérieux, Durham, NC).

PCR, cloning, and DNA sequencing. PCR primers are listed in Table 1. Primers used in an initial screen for *qnrB* were derived from an alignment of the first 20 *qnrB* alleles (http://www.lahey.org/qnrstudies) and those for *Citrobacter* 16S rRNA genes from an alignment of 21 *Citrobacter* gene sequences available in GenBank

(http://www.ncbi.nlm.nih.gov). PCR reactions were performed using genomic DNA prepared by boiling, PCR Supermix High Fidelity (Invitrogen, Carlsbad, CA), and temperatures appropriate for the nucleotide composition of the primers. Three *qnrB* alleles were cloned using vector pBC SK, digestion with endonuclease *Bam*H1 (New England Biolabs, Ipswich, MA), and selection on Mueller-Hinton agar with 25 µg/ml chloramphenicol. DNA sequencing was performed at the Tufts University Core Facility (Boston, MA). For all *qnrB* alleles both DNA strands were analyzed. GenBank accession numbers for *qnrB* alleles sequenced in this study are JN173050 (*qnrB13*), JN173051 (*qnrB17*), JN173052 (*qnrB27*), JN173053 (*qnrB29*), JN173054 (*qnrB32*), JN173055 (*qnrB33*), JN173056 (*qnrB34*), JN173057 (*qnrB35*), JN173058 (*qnrB36*), JN173059 (*qnrB37*), and JN173059 (*qnrB38*).

Plasmid transfer. The presence of transmissible resistance was evaluated by mating to *Escherichia coli* J53 Azi^R (8), selecting on Mueller-Hinton agar plates containing antimicrobial agents to which the *C. freundii* donor was resistant and 250 µg/ml sodium azide for counterselection.

***Citrobacter* species**. Citrobacter species were identified according to biochemical criteria (1, 10, 14) with tests for ornithine decarboxylase, and fermentation of malonate, raffinose, sucrose, and melibiose utilizing MicroScan® Neg ID Type 2 panels.

RESULTS

Since preliminary experiments indicated that *qnrB* was uncommon in isolates of *Citrobacter koseri* or *Citrobacter amalonaticus,* we collected clinical isolates belonging to the *Citrobacter freundii* complex from the clinical laboratories of the Lahey Clinic and the Massachusetts General Hospital (MGH). Seventy-seven percent came from urine

cultures. Eleven percent were resistant or intermediate in susceptibility to ciprofloxacin. The frequencies of nonsusceptibility for other antibiotics were sulfonamide (39%), trimethoprim (17%), ceftriaxone (13%), tetracycline (11%), gentamicin (10%), and kanamycin (0%).

Sixteen of 36 *Citrobacter* isolates from the MGH and 10 of 35 from the Lahey Clinic were positive by PCR for *qnrB* for a combined prevalence of 36.6%. None was positive for *qnrA, qnrC, qnrD,* or *qnrS*. Since all isolates tested susceptible to kanamycin, AAC(6')-Ib-cr (17) was also absent. To fully sequence the *qnrB* gene, primers bracketing the gene were used. PCR with primers psp2 and sc3 (Table 1) was positive with 9 strains, while PCR with primers psp2 and ds2 or ds3 was successful with 13 strains. Three strains amplified with neither primer pair and were sequenced after cloning their *qnrB* genes into plasmid pBC SK. One additional strain was negative with the screening primers but positive with a primer set amplifying a 216-bp *qnrB* segment. Subsequent studies showed that this strain contained a *qnrB* pseudogene with a substantial deletion.

Fifteen different *qnrB* alleles were detected, including 7 that are novel and have been assigned *qnrB32* to *qnrB38*. *qnrB9* was found in 8 strains, six from the MGH and two from the Lahey Clinic that could be further distinguished based on biochemical reactions . *qnrB12, qnrB27,* and new *qnrB35* were identified in 2 isolates each. In contrast to fully sensitive *C. freundii* strains with a ciprofloxacin MIC of 0.008-0.012 µg/ml, the ciprofloxacin MIC of *qnrB* positive strains ranged from 0.016 to ≥32 µg/ml with a median value of 0.094 µg/ml, with the value of ≥32 µg/ml not due to plasmid-mediated mechanisms, since an *E. coli* transconjugant with the *qnrB4* plasmid from the

clinical isolate with a ciprofloxacin MIC of ≥32 µg/ml had a MIC of only 0.19 µg/ml. All the new *qnrB* alleles were preceded by a LexA box (Table 2), allowing control by the bacterial SOS system (5, 23).

An amino acid alignment of the new QnrB alleles with others can be found at http://www.lahey.org/qnrstudies. Fig. 1 shows the relationship among QnrB alleles not shown to be plasmid-mediated and not known to occur except in *Citrobacter* spp. Several clusters are evident, and the new *qnrB* alleles differ little from *qnrB* varieties previously described in this genus. For example, in terms of amino acid differences, QnrB32 differs from QnrB13 by 2, QnrB33 from QnrB27 by 1, QnrB34 from QnrB12 by 1, QnrB35 from QnrB8 or QnrB25 by 5, QnrB36 from QnrB10 by 1, QnrB37 from QnrB12 by 2, and QnrB38 from QnrB8 by 3.

Two *Citrobacter* strains carried *qnrB* alleles known to occur in other *Enterobacteriaceae* and to be carried on transmissible plasmids. These strains, containing *qnrB2* or *qnrB4,* readily transferred their *qnrB* genes on multiresistant plasmids to *E. coli* J53 Azi^R. No *qnrB* transfer was detected from the remaining *qnrB* positive *Citrobacter* strains although 7 of 24 yielded potential transconjugants on selection with ampicillin, sulfonamide, or trimethoprim, a finding that suggests a chromosomal location for most of the *qnrB* alleles.

The *C. freundii* complex includes *C. braakii*, *C. werkmanii, and C. youngae,* species that can be distinguished from *C. freundii* by biochemical reactions (1). By such classification 5 of the 71 strains were *C. braakii*, three *C. werkmanii*, and three *C. youngae*. The remaining strains were classified as *C. freundii*. To differentiate the strains further, an 808-bp segment of the 16S rRNA gene of each isolate was amplified and

sequenced. Fig. 2 shows an alignment of the resulting sequence data with appended species identification by biochemical criteria, *qnrB* presence, and *qnrB* allele number. As noted by others, for citrobacter the correlation between 16S rRNA and biochemical characterization is imperfect (20). The three *C. werkmanii* clustered together and all contained *qnrB* alleles. Three of five *C. braakii* were *qnrB* positive, but the other two *C. braakii* were *qnrB* negative and unrelated by 16S rRNA. Some *qnrB* alleles were, however, closely linked to particular species, such as *qnrB12* with *C. werkmanii* and *qnrB27* with *C. braakii*. Such clustering again supports a chromosomal location for these *qnrB* genes.

Plasmid-mediated *qnrB* alleles are often associated with the gene-capturing element IS*CR1* and incorporated into integrons with other antibiotic resistance genes and an integrase gene, usually *intI1*. By PCR twenty one of 26 *qnrB+ Citrobacter* strains were negative for *intI1, intI2,* or *intI3* and 24 of 26 were negative for IS*CR1*. The only IS*CR1* positive strains were those containing *qnrB2* and *qnrB4* on transmissible plasmids. These strains were also integrase gene positive, as were single strains containing *qnrB9, qnrB12*, and *qnrB38*, but the *qnrB9* and *qnrB12* strains were among those transferring resistances other than quinolone resistance and hence could carry unrelated *intl* positive plasmids. The absence of IS*CR1* and *intI* strengthens the conclusion that in most *Citrobacter* isolates *qnrB* is chromosomal.

DISCUSSION

QnrB is more common in *Citrobacter* species than in other gram-negative bacteria. Park et al. reported a *qnr* prevalence of 38.4% in 138 strains of *C. freundii* from Korea, a frequency remarkably close to the 37% prevalence reported here, and higher

than they found in *Enterobacter cloacae*, *Enterobacter aerogenes* or S*erratia marcescens*. All but one of the *qnr* genes in the Korean *C. freundii* were *qnrB* with *qnrB2, qnrB1*, and *qnrB4* represented in order of frequency. Transmissibility was not studied (15).

While the first *qnrB* alleles to be described were clearly carried by conjugative plasmids (9), the majority of the *qnrB* alleles subsequently found in *Citrobacter* spp. have not been shown to be transferrable, while a chromosomal location has been proven in individual strains for *qnrB6, qnrB12,* and *qnrB16* by genome mapping with I-*Ceu*I and S1 nuclease followed by double hybridization for *qnr* and 23S rRNA genes (12, 19). The variety of *qnrB* alleles (thirty-eight) compared to the seven alleles for *qnrA*, five for *qnrS*, and one each for *qnrC* and *qnrD* is also consistent with a long-standing chromosomal location allowing sequence diversification.

A prolonged association with the *Citrobacter* chromosome can also account for the geographical dispersion of some alleles. Two of the *qnrB* alleles detected in our strains (*qnrB27* and *qnrB29*) were recently described in *Citrobacter* isolates from Korea (GenBank HM439641 and HM439649) and hence are widespread.

Additional evidence of a chromosomal location for *qnrB* is provided by the recent sequencing of *Citrobacter* genomes, several of which are available on line in various stages of completion. A segment of the draft genome of *C. youngae* ATCC 29220 (GenBank ABWL00000000) is shown in Fig. 3. A “pentapeptide repeat protein” occurs between a cluster of *psp* (phage shock protein) and *sap* (peptide ABC transporter, ATP-binding protein) genes. Nearby IS*CR1* or *IntI* genes are notably absent. The pentapeptide repeat protein is a new *qnrB* allele, here named *qnrB39.* The sequence of genes from

183 *pspD* to *sapC* in the *C. youngae* genome is the same as in a ~10-kb segment associated
184 with IS*CR1* and *IntI* genes in several *qnrB4* plasmids, with the addition in the plasmid
185 segments of an extra kb of apparently noncoding DNA between genes *cinA* and *ppp*.
186 Such *qnrB4* plasmids have been reported from Paris (22), two sites in China (11)
187 (GenBank EF683583), Singapore (EF682135), and Taiwan (FJ943500). If the extra kb of
188 DNA is ignored, there is 90.2 % identity between plasmid and *C. youngae* genome,
189 making this or the genome of a related *Citrobacter* the likely source of the plasmid
190 segment.

191 In this study *qnrB* alleles were identified using degenerate primers internal to the
192 *qnrB* gene and fully sequenced using primer pairs one of which was derived from *pspF*.
193 For strains with alleles *qnrB12, qnrB17, qnrB27, qnrB33, qnrB34, qnrB37*, and plasmid-
194 mediated *qnrB4* the second sequencing primers was derived from the *sdr* gene
195 downstream from *qnrB* in *C. youngae* ATCC 29220 or *qnrB4* plasmid DNA.

196 This primer pair did not, however, produce a PCR product with other alleles.
197 Another arrangement of genes is found with chromosomal *qnrB16* and plasmid-mediated
198 *qnrB2* (6, 9, 19). Both are bounded by *pspF* and *sapA*, but the open reading frame of a
199 gene of unknown function (Orf2 in (9)) occurs immediately downstream from these *qnrB*
200 alleles. Primers ds2 and ds3 were derived from the sequence of this downstream gene
201 and, combined with a primer from *pspF*, were used to sequence isolates making *qnrB9*,
202 *qnrB13*, *qnrB29*, *qnrB32*, *qnrB36* and plasmid-mediated *qnrB2*. Fig. 1 shows that the
203 *qnrB* alleles with downstream *sdr* or downstream *orf2* are more closely related to each
204 other than to alleles in the other group. Whether such clustering correlates with what are
205 ultimately designated as different *Citrobacter* species or sequence types is not yet known.

In a few strains neither primer pair yielded a product, and the *qnrB* genes were cloned for sequencing. Fig. 3 shows the resulting maps for *qnrB35* and *qnrB38*. Both were bounded by *pspF* and *sapA* but lack a downstream *sdr* or *orf2* site.

A *qnrB* gene is absent from other sequenced *Citrobacter* genomes. In the completed genome of *Citrobacter rodentium* ICC 168 (Gen Bank NC_013716) or the assembled genome of *C. freundii* ballerup 7851 (www.sanger.ac.uk), only about 100-bp are found between the end of *pspF* and the start of *sapA*. The genome of *C. koseri* ATCC BAA-895 (GenBank NC_009792) contains circa 1-kb of DNA in this position, but this DNA is unique to *C. koseri* and has no homology to *qnrB* or to any of the genes found between *pspF* and *sapA* in other genomes.

The prevalence, diversity, spread, and age of *qnrB* in *Citrobacter* (18), the lack of transmissibility of all but two of the *qnrB* genes detected in this study, the absence of gene capture elements IS*CR1* and *IntI* genes in most strains, the species specificity of particular alleles, and accumulating data from whole genome sequences all point to *Citrobacter* spp. as the likely origin of *qnrB*. Not all *Citrobacter* isolates, however, contain *qnrB*. Whether it confers an advantage in particular habitats or its presence implies membership in a taxonomic subgroup awaits further studies.

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TABLE 1. PCR Primers

Gene or site	Primer sequences (5' → 3')	Product size (bp)	Reference
qnrA	ATTTCTCACGCCAGGATTTG TGCCAGGCACAGATCTTGAC	573	(7)
qnrB	CTCTGGCRYTMGTYGGCGAA TTYGCBGYYCGCCAGTCGAA	504	This study
psp2[a]	AAATTTAAYCAGAAAAAAGC		This study
sc3[b]	GCTSARGAGAACAGCTATAC	972 with psp2	This study
ds2[c]	AAGAGTGGAAAATTTCCACA	914 with psp2	This study
ds3[d]	ATGGCTGAAGTTGAGATTAT	1068 with psp2	This study
qnrC	GGGTTGTACATTTATTGAATCG CACCTACCCATTTATTTTCA	307	(13)
qnrD	CGAGATCAATTTACGGGGAATA AACAAGCTGAAGCGCCTG	581	(3)
qnrS	ACTGCAAGTTCATTGAACAG GATCTAAACCGTCGAGTTCG	416	(7)
16S rRNA	TCTGAGAGGATGACCAGCCA GGGACTTAACCCAACATTTC.	808	This study
intI1,2,3	TGCGGGTYAARGATBTKGATTT CARCACATGCGTRTARAT		(24)
IS*CR1*	AAGGAACGCCACGGCGAGTCAA TGCAAAGACGCCGTGGAAGC	1167	(9)

[a]Matching sequence in *pspF* upstream from many *qnrB* alleles

[b]Matching sequence in short chain dehydrogenase/reducatase (*sdr*) downstream from *qnrB12* in GenBank AM77447

[c]Matching sequence in an unidentified gene (*orf2*) downstream from *qnrB*2 in GenBank AM234698

[d]Matching sequence downstream from *qnrB10* in GenBank EF636461

TABLE 2. Nucleotide sequence upstream from *qnrB* alleles. Components of a LexA binding site and the potential ATG initiation codon are shown in bold.

Allele	Sequence
qnrB9	ATGACGCCATTA**CTGT**ATAAAAAAA**CAG**GTACAAAT**ATG**GCT
qnrB12	ATGATGCAATCA**CTGT**ATAAAAAAA**CAG**GTTAATC**ATG**ATG
qnrB13	ATGACGCCATTA**CTGT**ATAAAAAAA**CAG**GTACAAAT**ATG**GCT
qnrB17	ATGACGCCATTA**CTGT**ATAAAAAAA**CAG**GTACAAAT**ATG**GCT
qnrB27	ATGATGAAATCA**CTGT**ATAAAAAAA**CAG**GTATATCATT**ATG**ACT
qnrB29	ATGACGCCATTA**CTGT**ATAAAAAAA**CAG**GTACAAAT**ATG**GCA
qnrB32	ATGACGCCATTA**CTGT**ACAAAAAAA**CAG**GTACAAAT**ATG**GCT
qnrB33	ATGATGAAATTA**CTGT**ATAAAAAAA**CAG**GTATATCATT**ATG**ACT
qnrB34	ATGATGCAATCA**CTGT**ATAAAAAAA**CAG**GTTAATC**ATG**ATG
qnrB35	ATTCCAGTAATA**CTGT**ATAAAAAAA**CAG**GCACATTATT**ATG**GCTC
qnrB36	ATGGCGTCATTA**CTGT**ATAAAAAC**ACAG**GCATAGAT**ATG**ACT
qnrB37	ATGATGCAATCA**CTGT**ATAAAAAAA**CAG**GTTAATC**ATG**ATG
qnrB38	ATCCCAGTAATA**CTGT**ATAAAAAAA**CAG**GCACATTATT**ATG**GCT

Figure legends

Figure 1. Amino acid alignment of those QnrB alleles found only in *Citrobacter* spp. and not shown to be carried by conjugative plasmids. The most closely related sequences cluster together.

Figure 2. *Citrobacter* strains aligned by similarity of 16S rRNA sequence with added species as determined by biochemical tests, presence of *qnrB* by PCR, and particular *qnrB* allele by sequencing. Those strains not identified as *C. braakii, C. werkmanii,* or *C. youngae* tested as *C. freundii*. R^+ indicates an allele mediated by a conjugative plasmid.

Figure 3. Genetic maps of chromosomal DNA from *C. youngae* ATCC 29220 (GenBank ABWL00000000), *C. freundii* ballerup 7851 (www.sanger.ac.uk), *C. rodentium* ICC168 (NC_013716), plasmid DNA from pRDDHA (AJ971344) and pJIBE401 (AJ609296), and DNA from strains F2503 containing *qnrB35* and S50552 containing *qnrB38*. Genes not identified in the text include *cinA* (competence/damage-inducible protein) and *ppp* (putative periplasmic protein).