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Citrobacter spp. as a Source of *qnrB* Alleles[▽]

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***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 1 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*.**

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 preantibiotic 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 was 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 medium 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 were derived from an alignment of 21 *Citrobacter* gene sequences available in GenBank (http://www.ncbi.nlm.nih.gov). PCRs 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 BamHI (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.

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

***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.

Nucleotide sequence accession numbers. 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*).

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 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 39% for sulfonamide, 17% for trimethoprim, 13% for ceftriaxone, 11% for tetracycline, 10% for gentamicin, and 0% for kanamycin.

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 were not amplified with either 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

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TABLE 1. PCR primers

Gene or site	Primer sequence (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[e]	This study
ds2[c]	AAGAGTGGAAAATTTCCACA	914[e]	This study
ds3[d]	ATGGCTGAAGTTGAGATTAT	1,068[e]	This study
qnrC	GGGTTGTACATTTATTGA ATCG CACCTACCCATTTATTTTCA	307	13
qnrD	CGAGATCAATTTACGGGG AATA AACAAGCTGAAGCGCCTG	581	3
qnrS	ACTGCAAGTTCATTGAACAG GATCTAAACCGTCGAGTTCG	416	7
16S rRNA	TCTGAGAGGATGACCAGCCA GGGACTTAACCCAACATTTC	808	This study
intI1, *intI2*, *intI3*	TGCGGGTYAARGATBTKG ATTT CARCACATGCGTRTARAT		24
IS*CR1*	AAGGAACGCCACGGCGAG TCAA TGCAAAGACGCCGTGGAAGC	1,167	9

[a] Matching sequence in *pspF* upstream from many *qnrB* alleles.
[b] Matching sequence in short-chain dehydrogenase/reductase (*sdr*) downstream from *qnrB12* in the sequence with GenBank accession number AM77447.
[c] Matching sequence in an unidentified gene (*orf2*) downstream from *qnrB2* in the sequence with GenBank accession number AM234698.
[d] Matching sequence downstream from *qnrB10* in the sequence with GenBank accession number EF636461.
[e] Size with psp2.

TABLE 2. Nucleotide sequence upstream from *qnrB* alleles

Allele	Sequence[a]
qnrB9	.....ATGACGCCATTA**CTGT**ATAAAAAAA**CAG**GT ACAAAT**ATG**GCT
qnrB12	.....ATGATGCAATCA**CTGT**ATAAAAAAA**CAG**GT TAATC**ATG**ATG
qnrB13	.....ATGACGCCATTA**CTGT**ATAAAAAAA**CAG**GT ACAAAT**ATG**GCT
qnrB17	.....ATGACGCCATTA**CTGT**ATAAAAAAA**CAG**GT ACAAAT**ATG**GCT
qnrB27	.....ATGATGAAATCA**CTGT**ATAAAAAAA**CAG**G TATATCATT**ATG**ACT
qnrB29	.....ATGACGCCATTA**CTGT**ATAAAAAAA**CAG**GT ACAAAT**ATG**GCA
qnrB32	.....ATGACGCCATTA**CTGT**ACAAAAAAA**CAG**GT ACAAAT**ATG**GCT
qnrB33	.....ATGATGAAATTA**CTGT**ATAAAAAAA**CAG**GT ATATCATT**ATG**ACT
qnrB34	.....ATGATGCAATCA**CTGT**ATAAAAAAA**CAG**GT TAATC**ATG**ATG
qnrB35	.....ATTCCAGTAATA**CTGT**ATAAAAAAA**CAG**GC ACATTATT**ATG**GCTC
qnrB36	.....ATGGCGTCATTA**CTGT**ATAAAAAC**CAG**GC ATAGAT**ATG**ACT
qnrB37	.....ATGATGCAATCA**CTGT**ATAAAAAAA**CAG**GT TAATC**ATG**ATG
qnrB38	.....ATCCCAGTAATA**CTGT**ATAAAAAAA**CAG**GC ACATTATT**ATG**GCT

[a] Components of a LexA binding site and the potential ATG initiation codon are shown in boldface.

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 seven that are novel and have been assigned *qnrB32* to *qnrB38*. *qnrB9* was found in eight strains, six from the MGH and two from the Lahey Clinic, that could be further distinguished on the basis of biochemical reactions. *qnrB12*, *qnrB27*, and the new allele *qnrB35* were identified in 2 isolates each. In contrast to fully sensitive *C. freundii* strains with ciprofloxacin MICs of 0.008 to 0.012 μg/ml, the ciprofloxacin MICs of *qnrB*-positive strains ranged from 0.016 to ≥32 μg/ml, with a median value of 0.094 μg/ml. The MIC of ≥32 μg/ml was 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. Figure 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

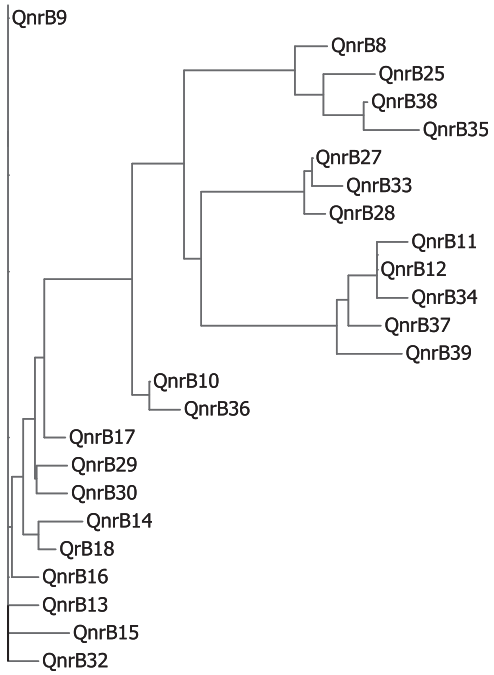

FIG. 1. Amino acid alignment of those QnrB alleles found only in *Citrobacter* spp. and not shown to be carried by conjugative plasmids. The most closely related sequences cluster together.

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 *C. freundii*. To differentiate the strains further, an 808-bp segment of the 16S rRNA gene of each isolate was amplified and sequenced. Figure 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 the 16S rRNA sequence and biochemical characterization is imperfect (20). The three *C. werkmanii* strains clustered together, and all contained *qnrB* alleles. Three of five *C. braakii* strains were *qnrB* positive, but the other two *C. braakii* strains were *qnrB* negative and unrelated by 16S rRNA sequencing. 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, 21 of 26 *qnrB*-positive *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 *intI*-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 South Korea, a frequency remarkably close to the 37% prevalence reported here and higher than the frequency that they found in *Enterobacter cloacae*, *Enterobacter aerogenes*, or *Serratia marcescens*. All but one of the *qnr* genes in the South Korean *C. freundii* strains were *qnrB*, with *qnrB2*, *qnrB1*, and *qnrB4* represented in that 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-CeuI and S1 nucleases, followed by double hybridization for *qnr* and 23S rRNA genes (12, 19). The variety of *qnrB* alleles ($n = 38$) compared to the 7 alleles for *qnrA*, 5 for *qnrS*, and 1 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 South Korea (GenBank accession numbers 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 online in various stages of completion. A segment of the draft genome of *C. youngae* ATCC 29220 (GenBank accession number 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 *pspD* to *sapC* in the *C. youngae* genome is the same as that in an ~10-kb segment associated with IS*CR1* and *intI* genes in several *qnrB4* plasmids, with the addition in the plasmid segments of an extra kb of apparently noncoding DNA between genes *cinA* and *ppp*. Such *qnrB4* plasmids have been reported from Paris, France (22), two sites in China (11) (GenBank accession number EF683583), Singapore (GenBank accession number EF682135), and Taiwan (GenBank accession number FJ943500). If the extra kb of DNA is ignored, there is 90.2% identity between plasmid and *C. youngae* genome sequences, making this or the genome of a related *Citrobacter* the likely source of the plasmid segment.

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

This primer pair did not, however, produce a PCR product with other alleles. Another arrangement of genes is found with chromosomal *qnrB16* and plasmid-mediated *qnrB2* (6, 9, 19). Both are bounded by *pspF* and *sapA*, but the open reading frame of a gene of unknown function (Orf2 in reference 9) occurs immediately downstream from these *qnrB* alleles. Primers ds2 and ds3 were derived from the sequence of this downstream gene and, combined with a primer from *pspF*, were used to sequence isolates making *qnrB9*, *qnrB13*, *qnrB29*, *qnrB32*, *qnrB36*, and plasmid-mediated *qnrB2*. Figure 1 shows that the *qnrB* alleles with downstream *sdr* or downstream *orf2* are more closely related to each other than to alleles in the other group. Whether such clustering correlates with what are ultimately designated 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. Figure 3 shows the resulting maps for *qnrB35* and *qnrB38.* Both are 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*

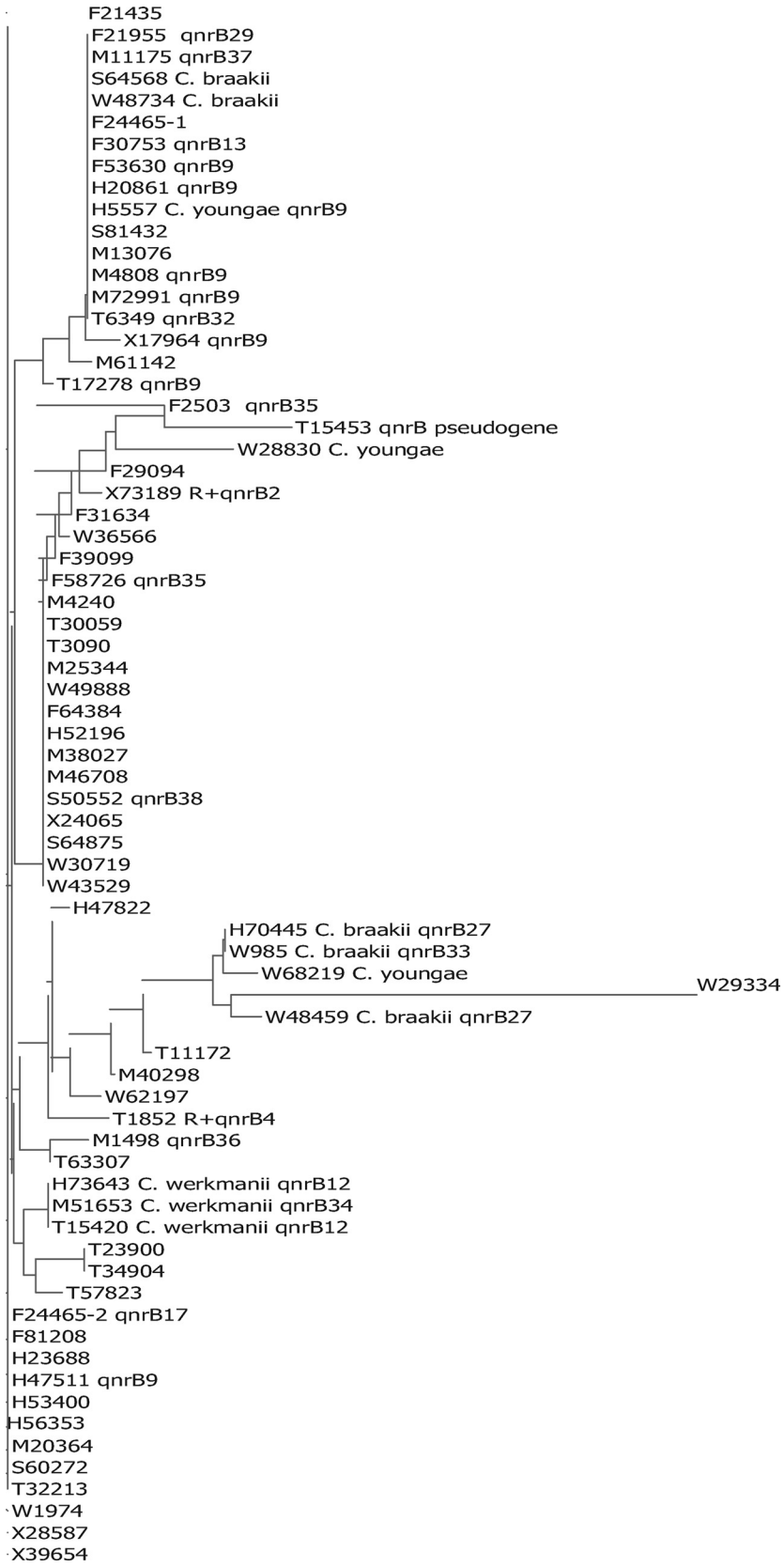

FIG. 2. *Citrobacter* strains aligned by similarity of 16S rRNA sequence with added species as determined by biochemical tests, presence of *qnrB* by PCR, and particular *qnrB* allele by sequencing. Those strains not identified as *C. braakii*, *C. werkmanii*, or *C. youngae* tested as *C. freundii*.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

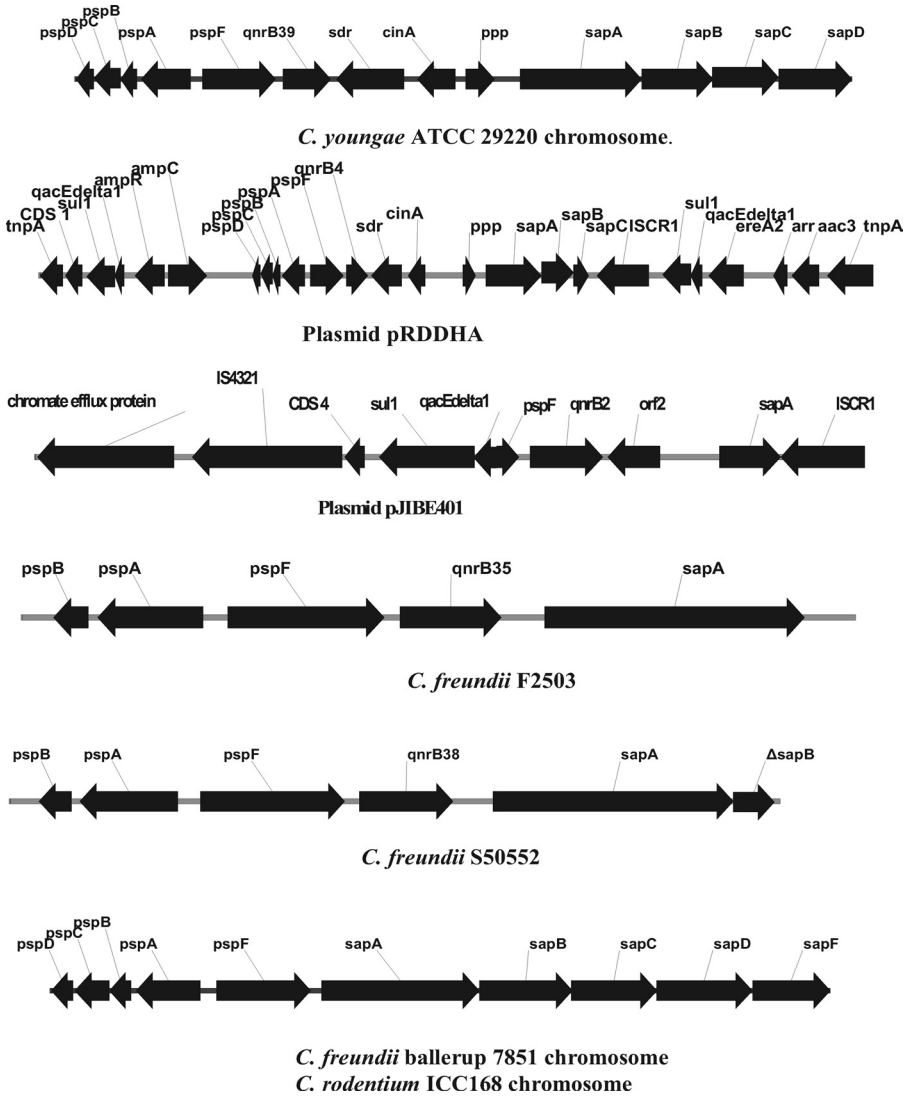

FIG. 3. Genetic maps of chromosomal DNA from *C. youngae* ATCC 29220 (GenBank accession number ABWL00000000), *C. freundii* ballerup 7851 (www.sanger.ac.uk), *C. rodentium* ICC168 (GenBank accession number NC_013716), plasmid DNA from pRDDHA (GenBank accession number AJ971344) and pJIBE401 (GenBank accession number 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).

ICC 168 (GenBank accession number NC_013716) or the assembled genome of *C. freundii* ballerup 7851 (www.sanger.ac.uk), only about 100 bp is 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 element 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 being 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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