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Phylogeny and Comparative Genomics Unveil Independent Diversification Trajectories of *qnrB* and Genetic Platforms within Particular *Citrobacter* Species

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To gain insights into the diversification trajectories of *qnrB* genes, a phylogenetic and comparative genomics analysis of these genes and their surrounding genetic sequences was performed. For this purpose, *Citrobacter* sp. isolates ($n = 21$) and genome or plasmid sequences ($n = 56$) available in public databases harboring complete or truncated *qnrB* genes were analyzed. *Citrobacter* species identification was performed by phylogenetic analysis of different genotypic markers. The clonal relatedness among isolates, the location of *qnrB* genes, and the genetic surroundings of *qnrB* genes were investigated by pulsed-field gel electrophoresis (PFGE), S1-/I-CeuI-PFGE and hybridization, and PCR mapping and sequencing, respectively. Identification of *Citrobacter* isolates was achieved using *leuS* and *recN* gene sequences, and isolates characterized in this study were diverse and harbored chromosomal *qnrB* genes. Phylogenetic analysis of all known *qnrB* genes revealed seven main clusters and two branches, with most of them included in two clusters. Specific platforms (comprising *pspF* and *sapA* and varying in synteny and/or identity of other genes and intergenic regions) were associated with each one of these *qnrB* clusters, and the reliable identification of all *Citrobacter* isolates revealed that each platform evolved in different recognizable (*Citrobacter freundii*, *C. braakii*, *C. werkmanii*, and *C. pasteurii*) and putatively new species. A high identity was observed between some of the platforms identified in the chromosome of *Citrobacter* spp. and in different plasmids of *Enterobacteriaceae*. Our data corroborate *Citrobacter* as the origin of *qnrB* and further suggest divergent evolution of closely related *qnrB* genes/platforms in particular *Citrobacter* spp., which were delineated using particular genotypic markers.

The *qnrB* genes constitute the most prevalent and diverse (>70 allelic variants; see http://www.lahey.org/qnrStudies/) group within the *qnr* family, encoding proteins responsible for decreased susceptibility to fluoroquinolones (1–4).

Some authors have proposed *Citrobacter* spp. as the origin of *qnrB* genes, mainly based on species distribution (>60% in *Citrobacter* spp., including isolates from the preantibiotic era), location (mostly on the chromosome), and the apparent absence of mobile genetic elements in the immediate genetic environment of *qnrB* genes, mostly by characterization of clinical *Citrobacter* sp. isolates (3–5). Nevertheless, the absence of correlation of *qnrB* genes with particular *Citrobacter* species, together with the lack of detailed characterization of *qnrB* platforms, hinders a clear establishment of the origin of *qnrB*. In fact, most of the methods conventionally used to identify *Citrobacter* spp. (biochemical or phenotypic features, matrix-assisted laser desorption ionization–time of flight mass spectrometry [MALDI-TOF MS], or 16S rRNA gene sequencing) (6–8) have low discriminatory power, hindering the accurate discrimination of these species.

Recently, Clermont et al. described a multilocus sequence analysis (MLSA) based on partial sequences of *rpoB* (β subunit of RNA polymerase gene), *pyrG* (CTP synthetase gene), *fusA* (protein synthesis elongation factor-G gene), and *leuS* (leucine tRNA synthetase gene) that allowed the discrimination of the 12 recognized *Citrobacter* species, namely, *Citrobacter freundii*, *C. amalonaticus*, *C. braakii*, *C. farmeri*, *C. gillenii*, *C. koseri*, *C. murliniae*, *C. rodentium*, *C. sedlakii*, *C. werkmanii*, *C. youngae*, and *C. pasteurii* (6).

In this work, we aim to gain insights into the diversification trajectories of *qnrB* within *Citrobacter* species and to unveil *qnrB* surroundings possibly involved in the dissemination of this gene to other *Enterobacteriaceae*. For that purpose, we performed an affiliation of *Citrobacter* species and *qnrB* genes described to date and a comparative analysis of genetic sequences surrounding *qnrB* using nonclinical *Citrobacter* sp. isolates and genome and plasmid sequences deposited in public databases.

MATERIALS AND METHODS

Bacterial isolates. Twenty-one nonclinical *Citrobacter* sp. isolates harboring *qnrB* genes recovered from different nonclinical origins, including untreated waters used for human consumption ($n = 12$; 2006 to 2008), ready-to-eat salads ($n = 3$; 2010), and trout aquaculture samples (trout, feed, and sediments from a river located upstream of the trout farm) ($n = 6$; 2010 to 2012) from different geographic regions in Portugal, were included in this study (see Table S1 in the supplemental material). The isolates carried *qnrB6* ($n = 1$), *qnrB9* ($n = 1$), *qnrB10* ($n = 3$), *qnrB17* ($n = 1$), *qnrB18* ($n = 1$), *qnrB56* ($n = 3$), *qnrB57* ($n = 2$), *qnrB58* ($n = 1$), *qnrB59* ($n = 3$), *qnrB72* ($n = 2$), *qnrB73* ($n = 1$), or truncated *qnrB*

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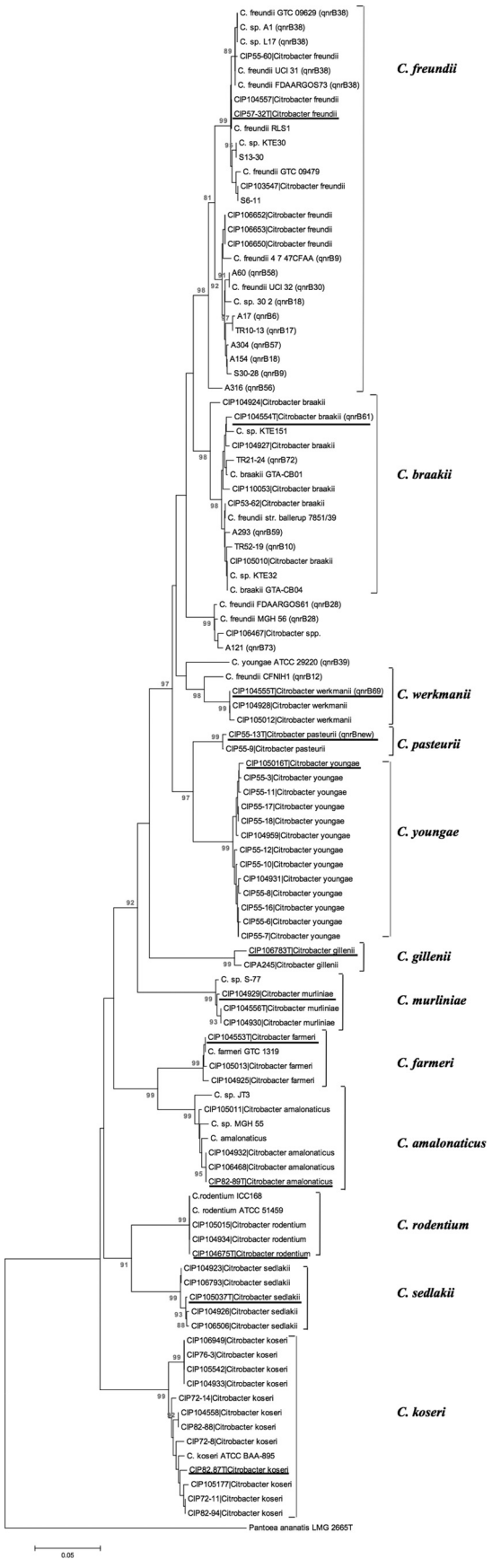


(Δ*qnrB*; $n = 2$) genes (9; P. Antunes, E. Machado, and L. Peixe, unpublished data) (see Table S1 in the supplemental material).

In addition, 40 *Citrobacter* sp. genomes and 16 *qnrB*-carrying plasmid sequences available from the Pathosystems Resource Integration Center (PATRIC) and/or the National Center for Biotechnology Information (NCBI) database were used for phylogenetic analysis and/or *qnrB* genetic surrounding comparisons.

Bacterial identification and phylogenetic analysis. Isolates included in this study were identified by biochemical methods (7), mass spectrometry (MALDI-TOF MS; Bruker Daltonik, Germany), and sequencing of 16S rRNA (8), *leuS* (leucine tRNA synthetase) (6), and *recN* (DNA repair protein) genes. PCR amplification and further sequencing of *recN* genes were performed by using primers recN-Fw (5′-ATTGCCATTGATGCTC TCGG-3′) and recN-Rv (5′-ANCGAGTCGGCCTGATCGT-3′) to amplify a 637-bp internal fragment and the following amplification conditions: one cycle of 3 min at 95°C; 35 cycles of 1 min at 95°C, 1 min at 56°C, and 1 min at 72°C; and 1 cycle of 1 min at 72°C.

Individual nucleotide sequences of genes included in the MLSA scheme of the *Citrobacter* genus (*rpoB*, *pyrG*, *fusA*, and *leuS*) (6) and *recN* were aligned and the average rates of similarity calculated using MEGA version 5.2.2 (http://www.megasoftware.net/) (10). The *leuS* gene sequences from Clermont et al. were included in this analysis (6). Similarity scores of the *leuS* and *recN* genes were calculated and individual phylogenetic trees were constructed in MEGA using the neighbor-joining (NJ) method (11), and genetic distances were calculated using the Kimura two-parameter model (12) in the case of nucleotide sequences and using the Jones-Taylor-Thornton (JTT) model (13) for LeuS and RecN protein sequences. The reliability of internal branches was assessed from bootstrap based on 1,000 resamplings (14). *Pantoea ananatis* strain LMG 2665^T was used as the outgroup.

Clonal relatedness. Clonal relationships among isolates belonging to the same species were established by pulsed-field gel electrophoresis (PFGE), using XbaI as a restriction enzyme and the following electrophoresis conditions: 10 to 40 s for 21 h at 14°C and 6 V/cm^2 (15). The criteria of Tenover et al. were used for comparison of band patterns obtained by PFGE, and isolates representing different PFGE-types were selected for the following studies (16).

Location, transferability, and phylogenetic analysis of *qnrB* genes. Location of *qnrB* genes was assessed by S1-/I-CeuI-PFGE and further hybridization with *qnrB* and 16S rRNA probes (17, 18). Conjugative transfer of *qnrB* was evaluated by broth and filter mating assays using *Escherichia coli* HB101 (streptomycin and azide resistant) as the recipient at a 1:2 donor-to-recipient ratio and selection plates containing ciprofloxacin (0.06 to 0.5 μg/ml) plus sodium azide (130 μm/ml) (19).

Affiliation within all *qnrB* genes described at the time of study design ($n = 74$; http://www.lahey.org/qnrStudies/) was generated as specified above for *leuS* and *recN* phylogenetic analysis.

Characterization of genetic surroundings of the *qnrB* genes. The genetic context of *qnrB* genes was characterized by PCR mapping (*pspF*, *sapA*, *intI1*, *intI2*, *intI3*, IS*Ecp1*, IS*3000*, IS*CR1*, IS*26*) and sequencing based on previously described sequences (3, 20–22). Sequences surrounding *qnrB* were further aligned and compared *in silico* with those deposited in the GenBank database using BLAST (http://blast.ncbi.nlm.nih.gov.sci-hub.org/Blast.cgi) and ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

FIG 1 Neighbor-joining (NJ) tree based on the comparison of *leuS* gene sequences of *Citrobacter* species analyzed in this study. Genetic distances were constructed using Kimura's two-parameter model. Numbers at branch points indicate bootstrap percentages (1,000 replications) from NJ analysis, and only values greater than 80% are shown. Horizontal bar, genetic distance of 0.05. *Citrobacter* species type strains are underlined, and the *qnrB* alleles are shown in parentheses. *Pantoea ananatis* strain LMG 2665^T was used as the outgroup (PATRIC fig|1378093.3.peg.2577). Please refer to Table S2 in the supplemental material for accession numbers of the sequences used.

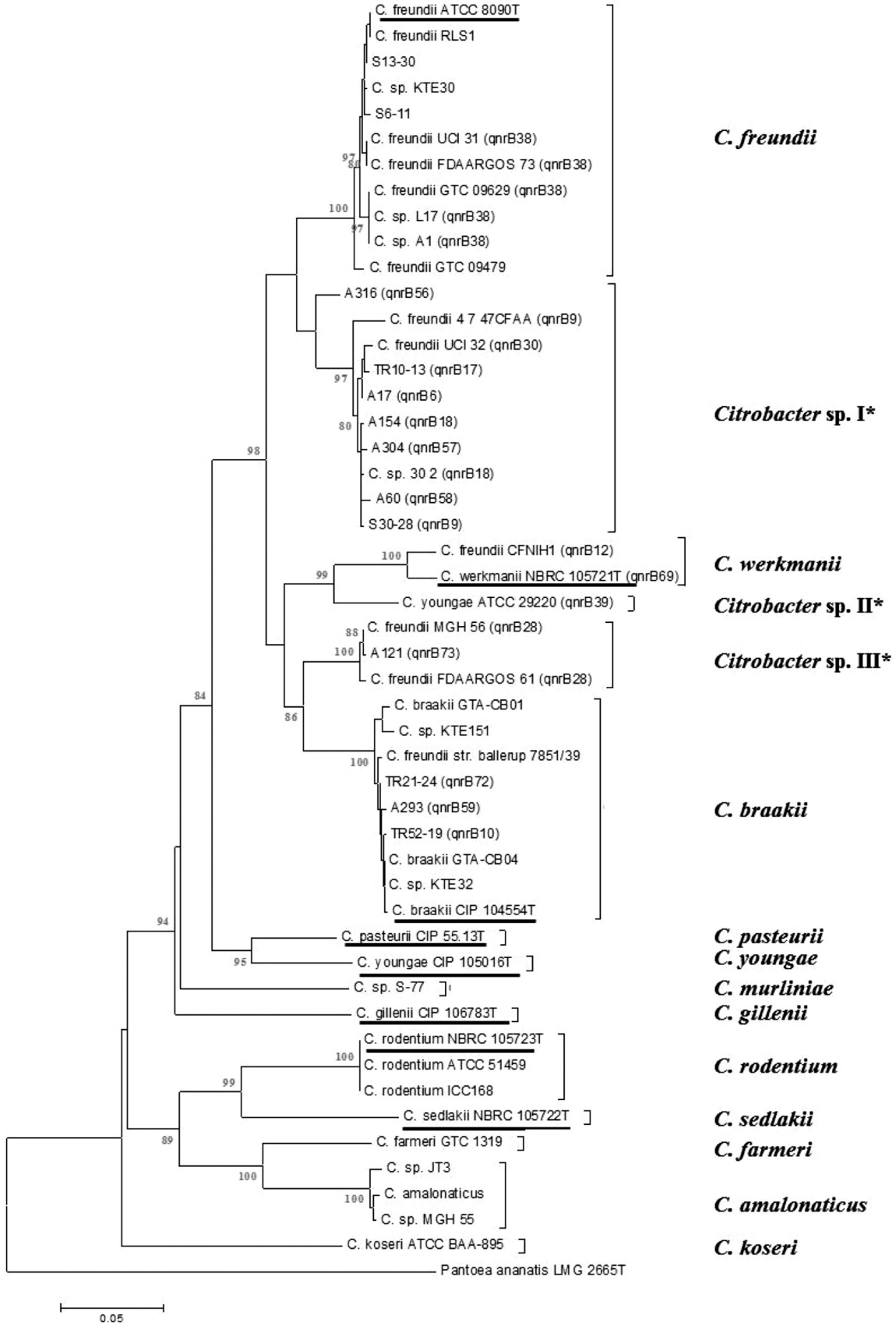
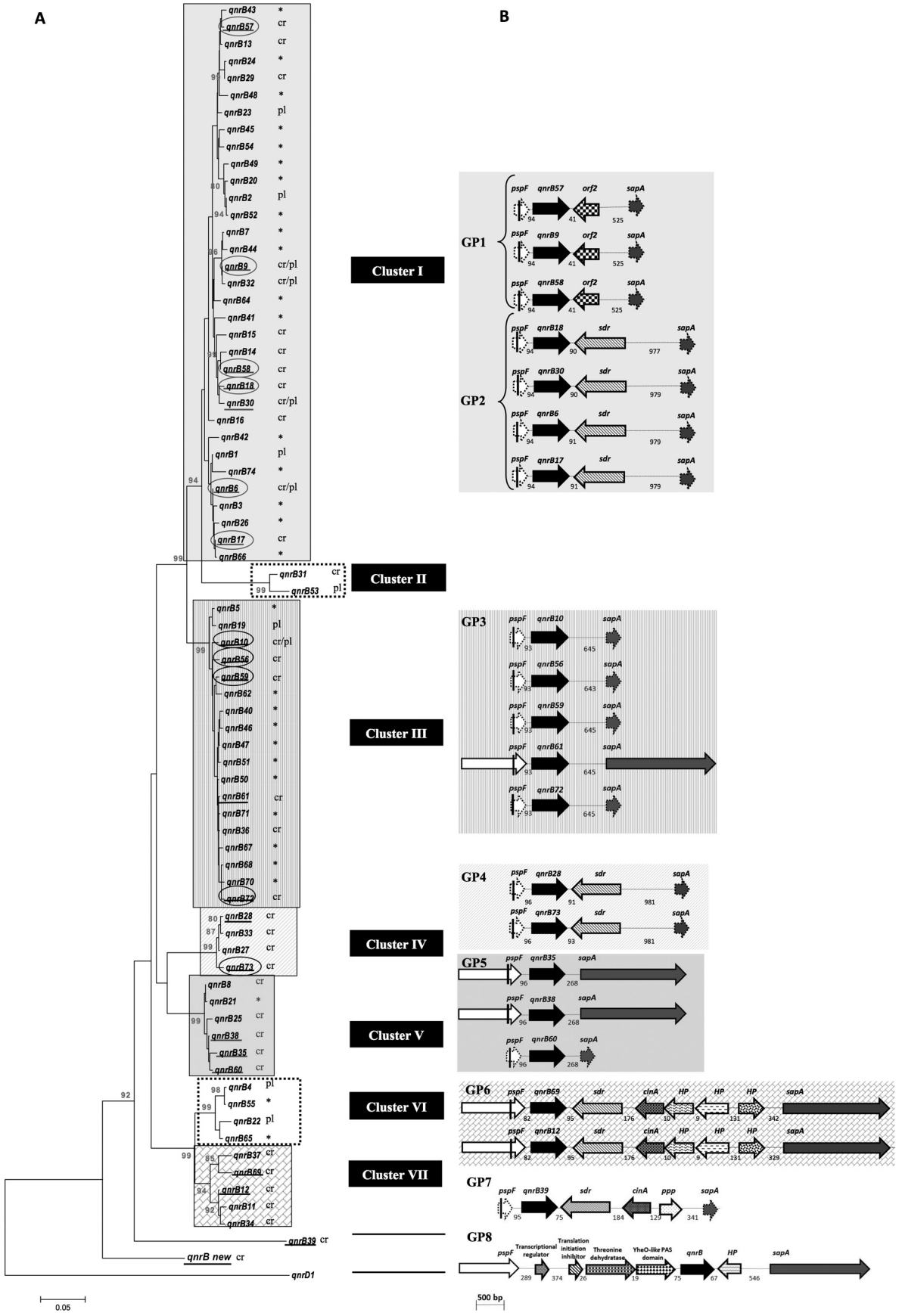


FIG 2 Neighbor-joining (NJ) tree based on the comparison of *recN* gene sequences of all *Citrobacter* species analyzed in this study. Genetic distances were constructed using Kimura's two-parameter model. Numbers at branch points indicate bootstrap percentages (1,000 replications) from NJ analysis, and only values greater than 80% are shown. Horizontal bar, genetic distance of 0.05. *Citrobacter* species type strains are underlined, and *qnrB* alleles are shown in parentheses. *, *Citrobacter* sp. I, *Citrobacter* sp. II, and *Citrobacter* sp. III correspond to putative novel species. *Pantoea ananatis* strain LMG 2665^T was used as the outgroup (PATRIC fig|1378093.3.peg.2577). Please refer to Table S2 in the supplemental material for accession numbers of the sequences used.


A
B
qnrB43 *
qnrB57 cr
qnrB13 cr
qnrB24 *
qnrB29 cr
qnrB48 *
qnrB23 pl
qnrB45 *
qnrB54 *
qnrB49 *
qnrB20 *
qnrB2 pl
qnrB52 *
qnrB7 *
qnrB44 *
qnrB9 cr/pl
qnrB32 cr/pl
qnrB64 *
qnrB41 *
qnrB15 cr
qnrB14 cr
qnrB58 cr
qnrB18 cr
qnrB30 cr/pl
qnrB16 cr
qnrB42 *
qnrB1 pl
qnrB74 *
qnrB6 cr/pl
qnrB3 *
qnrB26 *
qnrB17 cr
qnrB66 *
qnrB31 cr
qnrB53 pl
qnrB5 *
qnrB19 pl
qnrB10 cr/pl
qnrB56 cr
qnrB59 cr
qnrB62 *
qnrB40 *
qnrB46 *
qnrB47 *
qnrB51 *
qnrB50 *
qnrB61 cr
qnrB71 *
qnrB36 cr
qnrB67 *
qnrB68 *
qnrB70 *
qnrB72 cr
qnrB28 cr
qnrB33 cr
qnrB27 cr
qnrB73 cr
qnrB8 cr
qnrB21 *
qnrB25 cr
qnrB38 cr
qnrB35 cr
qnrB60 cr
qnrB4 pl
qnrB55 *
qnrB22 pl
qnrB65 *
qnrB37 cr
qnrB69 cr
qnrB12 cr
qnrB11 cr
qnrB34 cr
qnrB39 cr
qnrB new cr
qnrD1
0.05
Cluster I
Cluster II
Cluster III
Cluster IV
Cluster V
Cluster VI
Cluster VII
GP1
GP2
GP3
GP4
GP5
GP6
GP7
GP8
pspF qnrB57 orf2 sapA
pspF qnrB9 orf2 sapA
pspF qnrB58 orf2 sapA
pspF qnrB18 sdr sapA
pspF qnrB30 sdr sapA
pspF qnrB6 sdr sapA
pspF qnrB17 sdr sapA
pspF qnrB10 sapA
pspF qnrB56 sapA
pspF qnrB59 sapA
pspF qnrB61 sapA
pspF qnrB72 sapA
pspF qnrB28 sdr sapA
pspF qnrB73 sdr sapA
pspF qnrB35 sapA
pspF qnrB38 sapA
pspF qnrB60 sapA
pspF qnrB69 sdr cinA HP HP HP sapA
pspF qnrB12 sdr cinA HP HP HP sapA
pspF qnrB39 sdr cinA ppp sapA
pspF Transcriptional regulator Translation initiation inhibitor Threonine dehydratase YheO-like PAS domain qnrB HP sapA
500 bp

Nucleotide sequence accession numbers. The sequences of the genetic platforms associated with the different *qnrB* alleles characterized in this study have been deposited in the GenBank database under the accession numbers KP339254 (*qnrB6*), KP339255 (*qnrB9*), KP339256 (*qnrB10*), KP339257 (*qnrB17*), KP339258 (*qnrB18*), KP339259 (*qnrB56*), KP339260 (*qnrB57*), KP339261 (*qnrB58*), KP339262 (*qnrB59*), KP339263 (*qnrB72*), and KP339264 (*qnrB73*). *recN* and *leuS* nucleotide sequence data from the different *Citrobacter* sp. isolates identified in this study are available in the GenBank database under accession numbers KR998019 (*Citrobacter* sp. I), KR998020 (*Citrobacter* sp. I), KR998021 (*Citrobacter* sp. III), KR998022 (*Citrobacter* sp. I), KR998023 (*C. braakii*), KR998024 (*Citrobacter* sp. I), KR998025 (*Citrobacter* sp. I), KR998026 (*C. freundii*), KR998027 (*C. freundii*), KR998028 (*Citrobacter* sp. I), KR998029 (*Citrobacter* sp. I), KR998030 (*C. braakii*), KR998031 (*C. braakii*), KR998032 (*Citrobacter* sp. I), KR998033 (*Citrobacter* sp. I), KR998034 (*Citrobacter* sp. III), KR998035 (*Citrobacter* sp. I), KR998036 (*C. braakii*), KR998037 (*Citrobacter* sp. I), KR998038 (*Citrobacter* sp. I), KR998039 (*C. freundii*), KR998040 (*C. freundii*), KR998041 (*Citrobacter* sp. I), KR998042 (*Citrobacter* sp. I), KR998043 (*C. braakii*), and KR998044 (*C. braakii*).

RESULTS AND DISCUSSION

***Citrobacter* species identification and clonality.** The identification at the species level of all the *Citrobacter* sp. isolates included in this study was not possible by biochemical methods, MALDI-TOF MS, or sequencing of the 16S rRNA gene (data not shown), as previously recognized (6, 23). In contrast, analysis of *leuS* and *recN* gene sequences provided an accurate discrimination of the currently recognized *Citrobacter* species, as explained below.

The *leuS* gene presented the highest discriminatory power (average rate of similarity close to 88.5%, statistically supported) of the genes included in the MLSA scheme proposed by Clermont et al. (6). Therefore, the *leuS*-based phylogenetic tree allowed the delineation of 12 distinct clusters (Fig. 1), each one supported by a type strain from each *Citrobacter* species, corroborating the topology obtained by the concatenated affiliation of the MLSA scheme (6). These clusters were defined with a cutoff value of <97.5%, supported by bootstrap values greater than 92% (Fig. 1).

The *recN* gene provided a greater resolution than *leuS*, presenting an average rate of similarity close to 85.6%. The *recN* tree topology was overall congruent with that obtained for *leuS* sequences (Fig. 2), with the presence of the same 12 clusters observed (cutoff values of <96.1% statistically supported by bootstrap values greater than 94%) supported by sequences from the available type strains. Interestingly, 3 new clusters were observed, namely, *Citrobacter* sp. I ($n = 10$), *Citrobacter* sp. II ($n = 1$), and *Citrobacter* sp. III ($n = 3$), which might correspond to isolates from novel species (Fig. 2). *Citrobacter* sp. I presented a genetic distance of 0.071 (bootstrap value of 97%) with its closest related species *C. freundii*, whereas *Citrobacter* sp. II and *Citrobacter* sp. III presented genetic distances of 0.081 (bootstrap value of 99%) and 0.073 (bootstrap value of 100%) with the closest related species *C. werkmanii* and *C. braakii*, respectively. Further studies are in progress to clearly establish the identity of the isolates included in these clusters.

Phylogenetic trees constructed based on amino acid sequences of LeuS and RecN showed that most nucleotide substitutions were synonymous, despite resulting in a less clear delineation between species due to the higher conservative character of amino acid sequences (see Fig. S1 and S2 in the supplemental material).

According to our phylogenetic analysis, *Citrobacter* sp. isolates characterized in this study were identified as *C. braakii* ($n = 83$ PFGE types), *C. freundii* ($n = 22$ PFGE types), and putatively two novel species (*Citrobacter* sp. I [$n = 107$ PFGE types] or *Citrobacter* sp. III [$n = 11$ PFGE types]) (see Table S1 in the supplemental material).

Location and affiliation of *qnrB* genes. No plasmids were detected in any of the *Citrobacter* isolates included in this study, and in all cases, *qnrB* was chromosomally located and not transferable by conjugation, further supporting the natural occurrence of this gene in the chromosome of *Citrobacter* spp. (3–5). The *qnrB* gene diversity found was in accordance with previous data (24, 25), probably driven by the interplay of different selective events (natural recombination events and/or alternative selective forces) (1, 26–28).

The phylogenetic tree constructed based on *qnrB* gene sequences (Fig. 3A) revealed seven distinct clusters (I to VII) and two branches comprising *qnrB39* and a new *qnrB* (*C. pasteurii* strain CIP 55-13^T), supported by bootstraps of ≥92% and sharing ≤92.83% identity between them. The corresponding affiliation based on amino acid sequences of QnrB showed that most nucleotide substitutions were synonymous, which resulted in a similar tree topology (see Fig. S3 in the supplemental material), with some exceptions consisting of genes showing a higher degree of nucleotide divergence (*qnrB31*, *qnrB53*, or *qnrB39*), as observed by other authors for $bla_{CTX\text{-}M}$ genes (29). Our phylogenetic analysis also showed that most of the *qnrB* genes, including those characterized in this study, belonged to cluster I ($n = 33$, including *qnrB6*, *qnrB9*, *qnrB17*, *qnrB18*, *qnrB57*, and *qnrB58*) or to cluster III ($n = 18$, including *qnrB10*, *qnrB56*, *qnrB59*, and *qnrB72*), whose diversification might be favored by their association with particular host species and/or niches (see below). Few *qnrB* genes

FIG 3 Affiliation of *qnrB* genes and *qnrB* genetic platforms from *Citrobacter* spp. (A) Neighbor-joining tree based on 74 *qnrB* gene sequences (http://www.lahey.org/qnrStudies/). Genetic distances were constructed using the Kimura 2-parameter model. Numbers at branch points indicate bootstrap percentages (1,000 replications) from NJ analysis, and only values greater than 80% are shown. Horizontal bar, genetic distance of 0.05. The nucleotide sequence of *qnrD1* (GenBank accession number FJ228229) was used as the outgroup. The *qnrB* genes for which the genetic environment was first characterized in this study are surrounded by circles, whereas those available in the GenBank database are underlined. pl, plasmid-borne *qnrB*; cr, chromosomally located *qnrB*; *, *qnrB* location not assessed. (B) Schematic representation of the genetic platforms (GP) carrying chromosomally located *qnrB* genes. Numbers between ORFs indicate the size of the intergenic region in base pairs (bp). Vertical black bars represent IRR2. Genes identified in *qnrB* platforms are *pspF* (encoding a phage shock protein), *orf2* (open reading frame of a gene of unknown function), *sdr* (encoding a short-chain dehydrogenase/reductase protein), *cinA* (encoding competence/damage-inducible domain protein), *HP* (encoding a hypothetical protein), *ppp* (encoding putative periplasmic protein), and/or *sapA* (encoding a protein involved in antimicrobial peptide resistance). Genetic platforms have been deposited in the GenBank database under accession numbers KP339254 (*qnrB6*), KP339255 (*qnrB9*), ADLG01000026.1 (*qnrB9*), CP007557 (*qnrB12*), KP339256 (*qnrB10*), KP339257 (*qnrB17*), KP339258 (*qnrB18*), ACDJ02000027.1 (*qnrB18*), JMUJ01000007.1 (*qnrB28*), JTBV01000001.1 (*qnrB28*), JAPA01000008.1 (*qnrB30*), JN173057 (*qnrB35*), JN173060 (*qnrB38*), NZ_AMPE01000004.1 (*qnrB38*), NZ_AKTT01000018.1 (*qnrB38*), NZ_AOUE01000004.1 (*qnrB38*), JTBJ01000001.1 (*qnrB38*), JAPB01000002.1 (*qnrB38*), ABWL02000005.1 (*qnrB39*), KP339259 (*qnrB56*), KP339260 (*qnrB57*), KP339261 (*qnrB58*), KP339262 (*qnrB59*), AB734055 (*qnrB60*), AB734053 (*qnrB61*), BBMW01000005.1 (*qnrB69*), KP339263 (*qnrB72*), KP339264 (*qnrB73*), and CDHL01000019 (new *qnrB* from CIP 55-13^T).

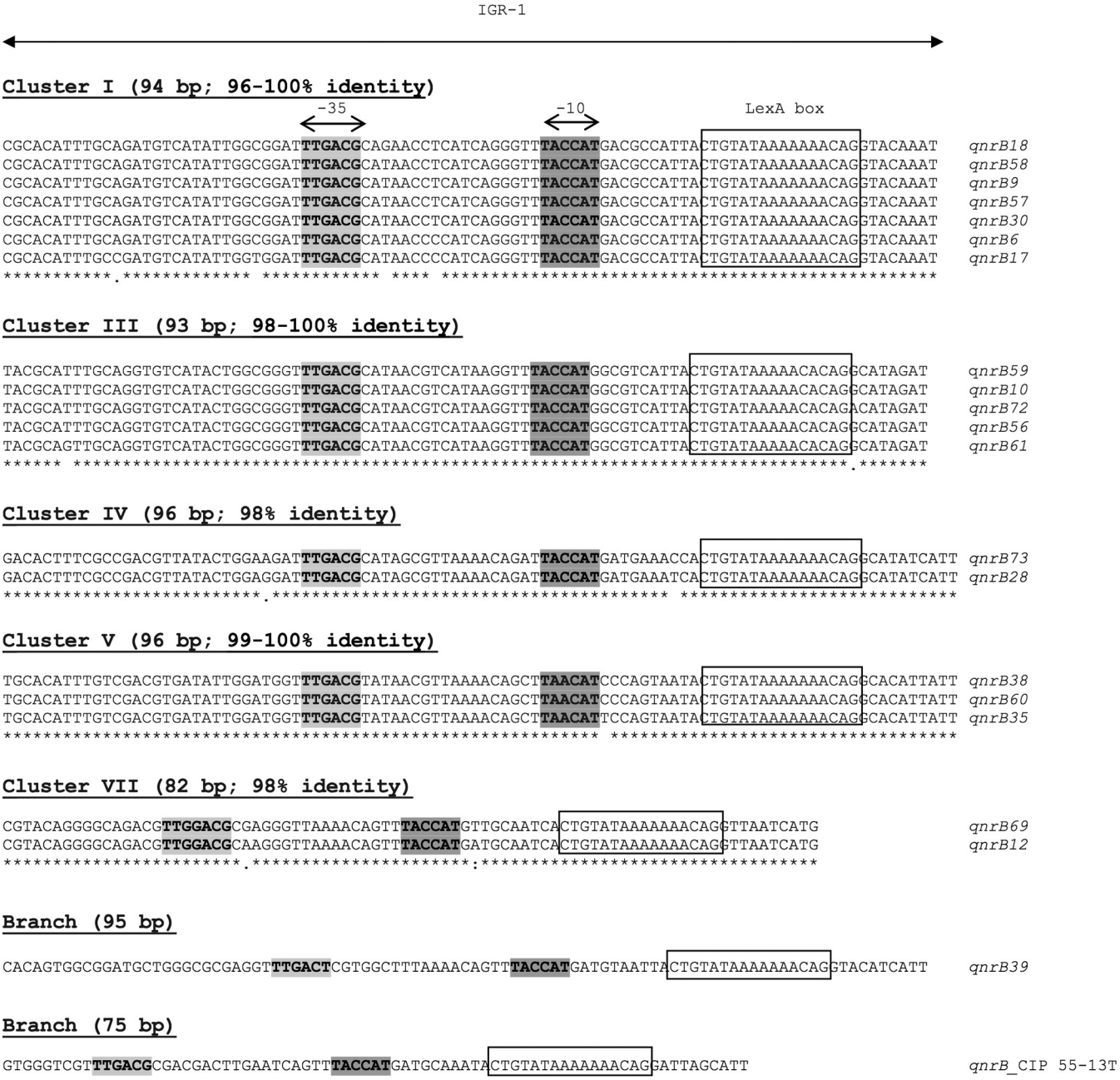


FIG 4 Nucleotide sequence alignment of intergenic regions upstream of chromosomally located *qnrB*. The −35 and −10 promoters are indicated by gray shading, and the sequence of the LexA box is boxed. Sequences were aligned using ClustalW2 software (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The IGR-1 sequences represented in this figure are found in the GenBank database through the accession numbers KP339254 (*qnrB6*), KP339255 (*qnrB9*), KP339256 (*qnrB10*), CP007557 (*qnrB12*), KP339257 (*qnrB17*), KP339258 (*qnrB18*), JMUJ01000007.1 (*qnrB28*), JAPA01000008.1 (*qnrB30*), JN173057 (*qnrB35*), JN173060 (*qnrB38*), ABWL02000005.1 (*qnrB39*), KP339259 (*qnrB56*), KP339260 (*qnrB57*), KP339261 (*qnrB58*), KP339262 (*qnrB59*), AB734055 (*qnrB60*), AB734053 (*qnrB61*), BBMW01000005.1 (*qnrB69*), KP339263 (*qnrB72*), KP339264 (*qnrB73*), and CDHL01000019 (new *qnrB* from CIP 55-13^T).

were enclosed in cluster II ($n = 2$), IV ($n = 4$, including *qnrB73*), V ($n = 6$), VI ($n = 4$), or VII ($n = 5$).

Detailed characterization of *qnrB* genetic platforms. Analysis of the genetic surroundings of complete *qnrB* genes revealed eight different *qnrB* genetic platforms (GP1 to GP8) (Fig. 3B). *pspF* (encoding a phage shock protein) and *sapA* (encoding a protein involved in antimicrobial peptide resistance) genes were consistently found upstream and downstream of *qnrB* genes, respectively. A high variability was observed mostly downstream of *qnrB*, with differences in the presence of other genes (*orf2*, *cinA*, *HP*, and/or *ppp*) and in the size and identity of intergenic regions (IGRs) upstream and downstream of *qnrB* (Fig. 3B). Interestingly, we observed conserved genetic platforms (gene content and sequence identity) for closely related *qnrB* genes (i.e., those grouped in the same cluster), with an exception in cluster I, possibly explained by a recombination event (Fig. 3).

As the characterization of IGRs was important to elucidate the origin and evolutionary routes of other antibiotic resistance genes (29, 30), we performed a detailed analysis of IGRs located in the *qnrB* genetic environment. In fact, the intergenic regions upstream of *qnrB* (IGR-1) were closely related (in size and in nucleotide sequence) among *qnrB* alleles that were grouped in the same cluster (identity, >96%) (Fig. 4), including those from cluster I (see above), whereas they exhibited a loss of identity between clusters (identity, 60% to 85%). This IGR-1 encompassed a LexA box consensus sequence located upstream of *qnrB* and downstream −35 and −10 promoter sequences (Fig. 4), which might directly regulate the expression of *qnrB* genes, as previously suggested (31, 32).

Interestingly, taking into consideration the similarity of the platforms carrying closely related *qnrB* genes and the identification of *Citrobacter* isolates carrying each *qnrB*, an association was found between each particular *qnrB* platform and specific *Citrobacter* species. The *qnrB* cluster I was associated with *Citrobacter* sp. I, *qnrB* cluster III with *C. braakii*, *qnrB* cluster IV with *Citrobacter* sp. III, *qnrB* cluster V with *C. freundii*, *qnrB* cluster VII with *C. werkmanii*, the branch comprising *qnrB39* with *Citrobacter* sp. II, and finally the branch comprising the new *qnrB* allele with *C. pasteurii*. One unique exception was detected (an isolate carrying *qnrB56* from cluster III belonged to *Citrobacter* sp. I instead of *C. braakii*), which may be explained by a genomic recombination event. This relationship was not established for *qnrB* alleles included in clusters II and VI due to the lack of genomic information from the corresponding strains in available databases. Thus, our findings provide additional data to support the acquisition of *qnrB* between *pspF* and *sapA* by a progenitor of at least some *Citrobacter* species prior to platform diversification. This hypothesis is further supported by the observation that 89% of isolates from particular species (*C. freundii*, *C. braakii*, *C. werkmanii*, *C. pasteurii*, *Citrobacter* sp. I, *Citrobacter* sp. II, and *Citrobacter* sp. III) carry a complete or truncated *qnrB* gene, suggesting species adaptation to variable ecological niches (see Table S2 in the supplemental material).

Analysis of the genetic environment surrounding the truncated *qnrB* genes (Δ*qnrB*) identified in this study revealed that the end of the *pspF-qnrB* intergenic region (encompassing promoter regions) and the first 360 bp of the *qnrB* gene were truncated (*pspF*-[47/49 bp]-Δ*qnrB*-[643 bp]-*sapA*). This genetic environment was identical (97% to 100%) with those described in the chromosome of other *Citrobacter* spp., including *C. freundii* strain ATCC 8090^T (GenBank accession numbers AB734052, AB734052, and AB734054), which suggests pseudogenization or deletion processes driven by insertion sequences (ISs) and eventually prophages (33, 34).

***In silico* analysis of *qnrB*-carrying plasmid platforms.** Our *in silico* analysis revealed that some of the *qnrB* genetic platforms identified in the chromosome of *Citrobacter* sp. I and *C. braakii* have already been detected in plasmids of different *Enterobacteriaceae* species (Fig. 1). This is the case for the genetic platforms containing *qnrB2*, *qnrB1*, or *qnrB6* (*qnrB* cluster I), previously identified in IncN, IncL/M, or IncFII plasmids in different *Enterobacteriaceae* species (GenBank accession numbers JX193301, JX101693, EU715254, KF193607, JX424423, JF775514, GU723682, and GU723680). Also, an identity was observed between the *qnrB10* platform detected in the chromosome of *C. braakii* and that in IncR plasmids (GenBank accession numbers EU052800, EU091084, and CP006662).

Some possibilities of mobilization of *qnrB* and/or regions surrounding *qnrB* were investigated. We did not find insertion sequences (ISs) or integrons in the *qnrB* genetic environment of the isolates characterized in this study, but an inverted repeat region (IRR; CTGAATTACTGGGT) was detected within the coding sequence of the *pspF* gene (including those associated with Δ*qnrB*). The IRR is also found in the same position in the chromosome of *Citrobacter* spp. (GenBank accession numbers AB734055, JN173060, AB734055, and AB734054) and in plasmids of different *Enterobacteriaceae* species (GenBank accession numbers EU523120, JN995611, JX101693, GU295957, JX424423, JX298080, and EU643617). This IRR is similar (0- to 5-bp mismatches) to IRR2, which was previously implicated in the mobilization of *qnrB19* after recognition by IS*Ecp1C* (35) and which might have been involved in the mobilization of other *qnrB* genes to plasmids. Nevertheless, different ISs (e.g., IS*26*, IS*CR1*, IS*Ecp1*, IS*3000*, IS*6100*) have been identified in the vicinity of diverse plasmid-mediated *qnrB* genes deposited in the GenBank database, suggesting the involvement of multiple mechanisms in the mobilization and/or assembly of the plasmid-associated *qnrB* genetic surroundings.

In conclusion, this study provides a comprehensive and extensive analysis of all *qnrB* genes and surrounding genetic platforms described to date and contributes to delineating the taxonomic positions of the different species within the *Citrobacter* genus. Our data corroborate *Citrobacter* as the origin of *qnrB* and further suggest independent diversification trajectories of specific *qnrB* genes/platforms in particular *Citrobacter* species (*C. freundii*, *C. braakii*, *C. werkmanii*, *C. pasteurii*, and in three putatively new *Citrobacter* species). Moreover, we unveil a potential route for mobilization of *qnrB* genes to plasmids, potentiating the dissemination of particular *qnrB* alleles in the clinical setting.

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