A Plasmid-Borne *Shewanella algae* Gene, *qnrA3*, and Its Possible Transfer In Vivo between *Kluyvera ascorbata* and *Klebsiella pneumoniae*©

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The plasmid-borne quinolone resistance gene *qnrA1* is prevalent in multidrug-resistant Enterobacteriaceae. A chromosomally encoded homologue in *Shewanella algae*, *qnrA3*, has been described. We isolated two *qnrA3*-positive strains, one of *Klebsiella pneumoniae* (He96) and one of *Kluyvera ascorbata* (Kas96), from the feces of an immunocompromised outpatient. The *qnrA3* allele was identical to that of *S. algae* except for 5 nucleotides and differed from *qnrA1* by 29 nucleotides affecting three amino acids. The analysis of the *qnrA3* genetic environment showed that *qnrA3* was inserted downstream from an ISCR1 element at a recombination crossover site described for other resistance genes, including *qnrA1*, and immediately upstream from IS26, a situation not described before. IS26 preceded an incomplete class 1 integron which contained, among other genes, *aac(6’)-Ib-cr*, another transferable quinolone resistance gene, and the β-lactamase gene *bla* 

Two novel mechanisms of resistance to fluoroquinolones in Enterobacteriaceae were recently described: quinolone acetylation mediated by AAC-(6’)-Ib-cr, an altered form of the original aminoglycoside 6’-N-acetyltransferase, and Qnr-mediated topoisomerase protection (40, 46). They differ markedly from the classical mechanisms, and their genes are plasmid rather than chromosome borne. The classical mechanisms comprise alterations in DNA gyrase and topoisomerase IV (the quinolone target), enhancement of drug efflux, a decrease in the permeability of the bacterial cell wall, or a combination thereof (14, 36).

The *qnr* genes known so far are *qnrA*, *qnrS*, and *qnrB* (38), with *qnrA* first described in *Klebsiella pneumoniae*, *qnrS* in *Shigella flexneri*, and *qnrB* in *K. pneumoniae* and *Escherichia coli* (20). Subsequent reports showed that these genes are also present in other species of Enterobacteriaceae, especially in multidrug-resistant isolates (6, 25, 32, 41). Variants were successively described for each gene, i.e., *qnrA1* to *qnrA6*, *qnrB1* to *qnrB9*, and *qnrS1* and *qnrS2* (2, 6, 20, 30, 37–39, 43). They have recently been reclassified, especially the *qnrB* alleles (17). While the amino acid identity among the proteins encoded by gene variants is between 91 and 99%, it is only 35 to 60% among *QnrA*, *QnrB*, and *QnrS*.

Transferable *qnr* genes are usually carried by large conjugative plasmids (50 to 180 kb) that often encode extended-spectrum β-lactamases (ESBLs) or AmpC-type β-lactamases (18, 30). The *qnr* genes were shown to be located in the vicinity of intact, antibiotic resistance determinant-containing class 1 integrons (20, 22, 26, 50). Transfer of plasmid-borne *qnr* was shown to occur by conjugation (19, 27, 50). Chromosome-borne *qnr*-type genes were discovered in environmental bacteria such as *Photobacterium profundum* (43), *Vibrionaceae* (9, 33, 43), and *Shewanella algae* (30, 34). In *S. algae*, which is hypothesized to be the origin of *qnrA*, the allele is highly homologous (90%) to *qnrA1*.

We recently screened strains of Enterobacteriaceae isolated in 2004 from the Hôpital Européen Georges Pompidou in Paris (France) for *qnrA* and found only two *qnrA*-positive isolates (one of *K. pneumoniae* and one of *Kluyvera ascorbata*). They were isolated from the feces of the same immunocompromised patient and possessed an original *qnrA* allele (GenBank accession no DQ435306) different from those in other strains isolated in Paris (6, 26). We found this allele to be homologous to *qnrA3* of *S. algae*, raising the question of how it had been transferred to and among the clinical isolates. It was the purpose of this study to analyze the genomic environment of *qnrA3* in both isolates and to evaluate its in vitro transferability.
TABLE 1. Oligonucleotides primers used for PCR and sequencing of the ca. 10-kb DNA fragment encompassing the qnrA3 gene in pHe96, pKas96, and Te He96/Ecin115

| DNA fragment (primer set) | Primer\(^a\) | Primer sequence (5’—3’)
|--------------------------|--------------|-----------------------------------
| qac\(\Delta E\)-qnrA3 \(A\) | qac\(\Delta E\) 1 s | GGCTTTTTTCTTGGTA TGGCA GCACCAAGCTGTGT
|                         | qnrA7 as      | CACTC CATTGTGCTGGGC AAGGAA
|                         | orf513 s      | TCAGTATCGTGGCT GCATG
|                         | orf513 s2     | GCAGCAACCGAGAA CGAGGCGC
|                         | sull1 s       | GAGCGGCACTGCGA GGTGTG
| qnrA3 \(B\)             | qnrA5 s       | GGGATGGAGATTTAT TGATAGAG
|                         | qnrA6 as      | CCTAAGGGAGCAGC CTATTA
|                         | qnrA9 s       | GCCATAAGATGTA CTCTGCT
|                         | qnrA10 as     | AGCGAGAGACATTAC CITATAGGC
| qnrA-qac\(\Delta E\) \(C\) | qnrA8 s       | GTCGAGATCTGTGGCC CTGGCA
|                         | qac\(\Delta E\) 1 as | CAGGCTTTTGCACCCAT GAAGC
|                         | orf513 1 s    | AGCTGCACTACCG TTTTCG
|                         | tnpA3 s       | GCCTACTTGACAA CAGTGC
|                         | int2 s        | GCGAAACCCTCATCC CGGGT
|                         | aac(6')-lb-cr s | GATGCTGGATAG TGCCATGC
|                         | blaOX1A3/30 s  | CCAGCTACTACGC AAACT
|                         | blaOX1A3/30 s2 | GGGCATCCTGAGC AGCCG
|                         | catB3 s       | GCAGTCCTACAAG AAACG
|                         | arr-3 s       | GGGCATCGGTAGC ACTGG
|                         | dfr s         | GCATGCTACATATA CATTAG
|                         | ant(3')-Ij-aac(6')-lb as | AGTGTGCAGGCGCT CAGG

\(^a\) Primers used for PCR are in bold; the other primers were used for sequencing, s, sense primer; as, antisense primer.

RESULTS AND DISCUSSION

The plasmid-borne qnrA3 allele and its genetic environment. *K. pneumoniae* He96 and *K. ascorbata* Kas96 were identified on the basis of their 16S rRNA sequences and particularly their *rpoB* sequences, which are more discriminatory in the identification of *Enterobacteriaceae* (28). Each strain contained a plasmid of ca. 70 kb (pHe96 and pKas96, respectively) (data not shown).

Since this is the first observation of *qnrA3* as a plasmid-borne gene, we analyzed in detail the sequences of *qnrA3* and its environment and compared them to published sequences of plasmids containing other *qnr* genes, such as pHSH2 from *qnrA1*-positive *E. coli* strains isolated in Hong-Kong (50), pQRI from a *qnrA1*-positive *E. coli* strain isolated in France (Paris) (26), and pOpKp311H from a *qnrA1*-positive *K. pneumoniae* strain isolated in Spain (Barcelona) (25).

The *qnrA* sequence was the same for *K. pneumoniae* He96 and *K. ascorbata* Kas96 and was 95.6% identical to that of *qnrA1* (46), with 29 nucleotide differences accounting for three amino acids.
acid substitutions, i.e., Arg39Gln, Ile108Val, and Ala127Thr. The deduced amino acid sequence was identical to that of the chromosome-borne \textit{qnrA3} gene of \textit{S. algae} (30), but there were five nucleotide differences (99.2% identity). No \textit{qnrB} or \textit{qnrS} gene was detected.

The sequences up- and downstream from \textit{qnrA3} are schematically shown in Fig. 1 and are detailed in Fig. 2. They were obtained from two fragments amplified on one hand with a sense primer in \textit{qacE}1/H9004 and an antisense primer in \textit{qnrA3} and on the other hand with a sense primer in \textit{qnrA3} and an antisense primer in \textit{qacE}1, with sequences being obtained using primers within the fragments (Table 1). Nucleotide sequences of the encompassing fragment of 10,776 bp from pHe96 and pKas96 were identical in He96 and Kas96, except for 541 bp which were absent in Kas96.

The \textit{qnrA3} gene was observed downstream from an \textit{IS} \textit{CR1} element similar to that described previously (45). The 233-bp sequence downstream from \textit{orf513} (M233 in Fig. 2) was identical to that of the chromosomal \textit{qnrA3} gene of \textit{S. algae} (30), but there were five nucleotide differences (99.2% identity). No \textit{qnrB} or \textit{qnrS} gene was detected.

The sequences up- and downstream from \textit{qnrA3} are schematically shown in Fig. 1 and are detailed in Fig. 2. They were obtained from two fragments amplified on one hand with a sense primer in \textit{qacE}1/H9004 and an antisense primer in \textit{qnrA3} and on the other hand with a sense primer in \textit{qnrA3} and an antisense primer in \textit{qacE}1, with sequences being obtained using primers within the fragments (Table 1). Nucleotide sequences of the encompassing fragment of 10,776 bp from pHe96 and pKas96 were identical in He96 and Kas96, except for 541 bp which were absent in Kas96.

The \textit{qnrA3} gene was observed downstream from an \textit{IS} \textit{CR1} element similar to that described previously (45). The 233-bp sequence downstream from \textit{orf513} (M233 in Fig. 2) was identical to those in most of the other \textit{qnrA1}-containing plasmids described so far (19, 21, 25, 26, 46, 50) and to that in pSAL-1 upstream from the \textit{ampC}-\textit{ampR} operon (48) (Fig. 2). In several \textit{qnrA1}-containing plasmids, such as pHSH2 and pOKp311H, \textit{ampR} was observed immediately downstream from \textit{qnrA1}, but this was not the case in our plasmids. This suggests that the insertion of \textit{qnrA3} downstream from \textit{IS} \textit{CR1} may have followed the same mechanism as that of \textit{qnrA1} and also as that of the insertion of the chromosome-borne region encoding \textit{ampC}-\textit{ampR} from \textit{Morganella morganii}. Indeed, the insertion probably occurred at the recombination crossover site (ACCC-) at the 3' end of \textit{IS} \textit{CR1} (Fig. 2), as described previously (1, 48).

The sequence immediately downstream from \textit{qnrA3} was identical to that found downstream from \textit{qnrA3} in the \textit{S. algae} chromosome (34), which confirms that \textit{qnrA3} has been excised from chromosomal DNA of \textit{S. algae} or similar organisms, and was ended by a 7-bp element (M7) found in the other \textit{qnr}-containing plasmids and also in pSAL-1. After 124 bp (M124) that were 92% identical to pHSH2 and other \textit{qnrA1}-containing plasmids but of unknown origin, there was an unusual insertion of an IS26 element (11). Proximity of IS26 and \textit{qnrA1} was recently reported for pOKp311H (25).

Downstream from IS26, 52 bp were identical with those found in pC15-1a, a multiresistance plasmid containing \textit{bla}_{CTX-M-15} and described from an \textit{E. coli} outbreak in Canada (5) (Fig. 3). Sequence identity with pC15-1a ended at the site of insertion of an incomplete \textit{intI} gene and resumed with the succession of resistance gene cassettes [\textit{aac(6')-Ib-cr} to \textit{catB3}] (Fig. 1 and 3).

The same sequence organization was also found in In37, present in pHSH2 (Fig. 1) (50). Compared to In37, a 217-bp deletion was observed in pHe96 upstream from \textit{aac(6')-Ib-cr}, which included the 83-bp 5' terminus of \textit{intI} (Fig. 3). The \textit{intI} gene also lacked 81 bp at its 3' end. The 217-bp deletion accounted for the absence of start and stop codons in \textit{intI} and of the promoter \textit{P}_{int} while the promoter \textit{P}_{c} was complete (Fig. 3). However, the −10 box of the promoter \textit{P}_{c} differed from

FIG. 1. Genetic environment of \textit{qnrA3} in \textit{K. pneumoniae} He96 (pHe96) and in \textit{K. ascorbata} Kas96 (pKas96). The 59-bp elements are indicated by circles. The sequences of pHe96 are compared with those described for pHSH2 (50) (GenBank accession number AY259086) and pC15-1a (5) (GenBank accession number NC005327).
In37 by two mutations and was identical to that found in pCTBacter-M-3, isolated from a multidrug-resistant strain of Citrobacter freundii (31). Since the DNA segments comprising IS26–aac(6‘)-Ib-cr in pHe96 were found adequately upstream from aac(6’)–Ib-cr–tnpA26 in pHe96, although in pC15-1a neither gene is present as an integron–rearrangement due to IS26, this may have been derived from pC15-1a.

**Characterization of antibiotic resistance determinants.** K. pneumoniae He96 exhibited a β-lactam resistance phenotype conferring resistance to piperacillin combined with tazobactam (10), whereas K. pneumoniae He96 harbored one qnrA gene; 19 bp homologous to the S. algae chromosome; M7 (detailed above); M124, common to pHSIH2 and pQP311H; and IS26, composed of a complete tnpA gene (717 bp) and the specific 51-bp inverted repeat.

Plasmids of ca. 70 kb containing qnrA3 were transferred at a frequency of 5 x 10^-6. The MICs of cefepime and ceftaxime, as well as the ratios of the MICs for cefepime divided by the MICs for ceftaxime, were determined. The MICs for ceftaxime were higher than those for cefepime with both strains harbored.

**Figure 2.** Schematic map (not to scale) of the regions upstream and downstream from qnrA3 and comparison with published qnrA1-containing plasmids (pHSIH2 [50]; pQR1 [26]; pQP311H [25]) and pSAL-1, containing DNA sequences of M. morganii from that in In37, at variance with the gene organization in pC15-1a (Fig. 1). Consequently, pHe96 cannot be derived from In37 but may have been derived from pC15-1a.

**Characterization of antibiotic resistance determinants.** K. pneumoniae He96 exhibited higher levels of resistance to quinolones than K. ascorbata Kas96 (Table 2). Since qnrA3 was reported to confer only decreased susceptibility to quinolones (34) and the MICs of ciprofloxacin and of levofloxacin were high in K. pneumoniae He96, we sought an additional quinolone resistance mechanism such as topoisomerase mutation. K. pneumoniae He96 harbored one qnrA mutation (Ser83Phe), with no mutation in gyrB or in the topoisomerase IV genes. This qnrA mutation added to the quinolone resistance phenotype conferred by the two plasmid-borne quinolone resistance genes, qnrA3 and aac(6’)-Ib-cr.

K. pneumoniae He96 exhibited a β-lactam resistance phenotype including resistance to piperacillin as well as combinations of clavulanic acid with amoxicillin or ticarcillin and a decrease in susceptibility to piperacillin combined with tazobactam, ceftaxime, and cephalosporins, as previously described for OXA-1/30 producers (10), whereas K. ascorbata appeared to be susceptible to amoxicillin-clavulanic acid, piperacillin-tazobactam, ceftaxime, and cephalosporins. However, both strains harbored blaOXA-1 and produced an enzyme compatible with an OXA-type β-lactamase (data not shown). In addition, in He96 we found an SHV-1 variant (Leu35Gln, Thr149Ser) not previously described and different from the chromosomal gene; M7 (detailed above); M124, common to pHSIH2 and pQP311H; and IS26, composed of a complete tnpA gene (717 bp) and the specific 51-bp inverted repeat.
frequency of $10^{-2}$ from *K. pneumoniae* He96 or *K. ascorbata* Kas96 to *E. coli* J53. This frequency is among the highest reported for other plasmid-borne *qnr* alleles (19, 49). The *blaOXA-1/30* gene was cotransferred along with tetracycline and trimethoprim resistance determinants. Transfer of *aac(6')-Ib-cr* was first assumed on the basis of increased MICs of kanamycin and tobramycin for the *E. coli* transconjugants (Table 2). Since similar MICs were observed in both transconjugants (Table 2), the expression of the *aac(6')-Ib-cr* gene from the two plasmids is probably similar, despite the differences in its surrounding se-

TABLE 2. MICs of quinolones and aminoglycosides, and susceptibility phenotypes for other antibiotics, for *K. pneumoniae* He96, *K. ascorbata* Kas96, *E. coli* J53, *E. cloacae* Ecl115, and their respective *qnrA3*-positive transconjugants

<table>
<thead>
<tr>
<th>Species and strain</th>
<th>Quinolones</th>
<th>Aminoglycosides</th>
<th>Susceptibility phenotype*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC (µg/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NAL</td>
<td>NOR</td>
<td>CIP</td>
</tr>
<tr>
<td><em>K. pneumoniae</em> He96</td>
<td>128</td>
<td>32</td>
<td>8</td>
</tr>
<tr>
<td><em>K. ascorbata</em> Kas96</td>
<td>64</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td><em>E. coli</em> J53</td>
<td>4</td>
<td>0.03</td>
<td>0.008</td>
</tr>
<tr>
<td>Te He96/J53</td>
<td>16</td>
<td>1</td>
<td>0.25</td>
</tr>
<tr>
<td>Te Kas96/J53</td>
<td>8</td>
<td>0.5</td>
<td>0.12</td>
</tr>
<tr>
<td><em>E. cloacae</em> Ecl115</td>
<td>4</td>
<td>0.06</td>
<td>0.01</td>
</tr>
<tr>
<td>Te He96/Ecl115</td>
<td>8</td>
<td>0.5</td>
<td>0.25</td>
</tr>
<tr>
<td>Te Kas96/Ecl115</td>
<td>8</td>
<td>0.5</td>
<td>0.25</td>
</tr>
</tbody>
</table>

*a* NAL, nalidixic acid; NOR, norfloxacin; CIP, ciprofloxacin; LVX, levofloxacin; MXF, moxifloxacin; GAT, gatifloxacin; GEN, gentamicin; KAN, kanamycin; TOB, tobramycin; AMK, amikacin.

*b* TMP, trimethoprim; TET, tetracycline; PIP, piperacillin; TZP, piperacillin-tazobactam; R, resistant; I, intermediate; S, susceptible.
quences mentioned above. Furthermore, the presence of this gene, which has the peculiar property of conferring selective resistance to quinolones with a nonsubstituted piperezinyl group at C-7, may explain why the transconjugants of E. coli 353 showed a greater increase in the MICs of ciprofloxacin and norfloxacin (16- and 32-fold increases) than in those of levofloxacin, moxifloxacin, and gatifloxacin (2- to 16-fold) in comparison to parental strains. The second aminoglycoside resistance gene in the 10-kb fragment, _ant(3')-Ia-aac(6')-Ib_, was truncated at its 3' end for the last 585 bp and thus was not assumed to confer additional aminoglycoside resistance.

For the _E. coli_ 353 transconjugants obtained from the parental strains He96 and Kas96, PCR-based replicon typing was positive for N-type replicons and negative for the other types, which suggests that _qnrA3_ is harbored on an IncN plasmid. _qnrA1_ genes have been associated so far with the IncA/C-type plasmids but not with IncN-type plasmids (35). Conversely, IncN-type plasmids were previously associated with the _β-lactamase genes bla_VIM-1_ (8) and _blaCTX-M-3_ (15) but not with _qnr_ genes, although replicon typing was seldom done in _qnr_-positive strains.

**Interspecies transfer of qnrA3.** In light of the observation of the _qnrA3_ variant, so far described only for _Shewanella_ (30), on two close-to-identical ca. 10-kb plasmid-borne fragments in isolates of different bacterial species from the same patient, we suspected the possibility of an in vivo interspecies transfer of _qnrA2_. We therefore tried to reproduce the presumptive in vivo transfer from _K. ascorbata_ Kas96 or _K. pneumoniae_ He96 to other clinical isolates of _Enterobacteriaceae_. A _qnrA-negative E. cloacae_ strain that was susceptible to tetracycline and trimethoprim, thereby allowing for the selection of the _qnrA3_ plasmid-containing transconjugants, was successfully conjugated. However, the transfer was observed at a frequency of 10^{-5}, i.e., 1,000-fold lower than that between _K. ascorbata_ and _E. coli_ 353. The low frequency of transfer may be due to inefficient conjugation or to the occurrence of recombination within the host plasmid (the strain was an ESBL producer) mediated by integron-like structure (3).

To check this hypothesis, we first compared the plasmid contents in _E. cloacae_ Ecl115 and in the two transconjugants _E. cloacae_ Tc He96/Ecl115 and _E. cloacae_ Tc Kas96/Ecl115. In _E. cloacae_ Ecl115, the presence of two plasmids of the IncHI2 and IncL/M types, but none of the IncN type, was suspected on the basis of replicon typing results. The IncHI2 type has previously been associated with _blaCTX-M-3_ (15), while to our knowledge an association of the L/M group with antibiotic resistance genes has not been reported. The _E. cloacae_ transconjugants were indeed positive for both H12 and L/M replicons and also for the N-type replicon corresponding to the plasmid from strains He96 and Kas96.

We also studied _E. cloacae_ Ecl115 for genes similar to those included in _pHe96_ and the two transconjugants for additional genes in the same order as in the 10-kb fragment containing _qnrA3_. In _E. cloacae_ Ecl115, we detected an IS26 element, similar to that of _pHe96_ and _pKas96_, with a partial _aac(3’)-II_ gene upstream from IS26, in the same genetic context as described for _pC15-1a_ (5) but downstream from an _ISCR1_ element in the case of _E. cloacae_ Ecl115. The ESBL gene was _blaCTX-M-3_ - a gene shown to originate from the _K. ascorbata_ chromosome and from which _blaCTX-M-15_ contained in _pC15-

1a, was derived by a point mutation leading to _Asp240Gly_ (42). Sequencing of the 10-kb fragment amplified from the transconjugant Tc He96/Ecl115 revealed sequence identity with _pHe96_, including _qnrA3_. Overall, this favors the hypothesis of a conjugative transfer between two strains of clinical origin, strains which usually are more difficult to conjugate than laboratory strains (50).

**K. ascorbata** is an environmental, waterborne bacterium that may cause food-borne infections in humans (13). Infections due to _K. ascorbata_ have rarely been described but may occur in immunocompromised patients such as the patient with Hodgkin’s disease from whom strain Kas96 was isolated. In this patient, the strain did not cause infection; however, it was found to be dominant in his gut flora, together with _K. pneumoniae_. The patient had received β-lactams and vancomycin but not quinolones during the month before the isolation of _K. pneumoniae_ He96 and _K. ascorbata_ Kas96. The role of _K. ascorbata_ as a reservoir of resistance genes has been recognized with the discovery, in their chromosomes, of a variety of _CTX-M_ genes (16, 23, 42). Since _K. ascorbata_ may live for long periods as a commensal in the human gut, it may well contribute to resistance gene transfer by conjugation to other inhabitants of this ecosystem, transient or not, such as _K. pneumoniae_ and _E. cloacae_ (13).

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