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BRIEF COMMUNICATION

Characterization of a novel *qepA3* variant in *Enterobacter aerogenes*



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quinolone resistance

Abstract Five isolates harboring *qepA* were studied by polymerase chain reaction (PCR) amplification and relevant methods. One was determined to be a novel *qepA3* from *Enterobacter aerogenes*, and four involved three *qepA1* and one *qepA2* determinants from *Escherichia coli*; the *qepA3* changed five amino acids. These results characterized genetic structures A, B, C, D, and E. Copyright © 2016, Taiwan Society of Microbiology. Published by Elsevier Taiwan LLC. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Introduction

In the past, the most common mechanisms of resistance to quinolones for pathogenic bacteria were the genetic mutations in chromosomal encoding DNA gyrase and/or topoisomerase IV,^{1,2} and changes in the expression of porin proteins for efflux pumps such as *qepA* and *oqxAB* were also common quinolone resistance mechanisms.³ Recently, a new plasmid mediated quinolone efflux pump, *qepA1*, was identified in three *Escherichia coli* isolates from Japan and Belgium.^{4,5} The *qepA1* gene was associated with the *rmtB* gene, which conferred high level resistance to aminoglycosides.^{6,7} In the course of study, we discovered a novel

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qepA3 variant, three *qepA1*, and one *qepA2* determinants in an isolate of *Enterobacter aerogenes* and four isolates of *E. coli*, and reported features of the *qepA* genes.

Materials and methods

Strains

Ninety-eight consecutive non-duplicate isolates including *E. coli* (43 isolates), *E. aerogenes* (36 isolates), and *Enterobacter cloacae* (19 isolates) were collected and detected for *qepA* from specimens in our laboratories between January 2008 and June 2010. Five of 98 isolates carried the *qepA* gene by polymerase chain reaction (PCR) amplification and sequencing. The numbers EC2468, EC2751, EC3762, EC3319, and EA3201 represented these four isolates of *E. coli* and one isolate of *E. aerogenes*, respectively. All isolates were identified with Vitek GNI⁺ cards (bioMérieux, Lyon, France), and species identification was confirmed by sequence analysis of the 16 S-23 S rRNA gene intergenic spacer region.

Conjugation experiments

Conjugation experiments were performed with five isolates as donors and azide-resistant *E. coli* J53 as recipient strain by filter mating. Transconjugants were selected on LB agar plates supplemented with sodium azide (150 µg/mL) and ciprofloxacin (0.25 µg/mL). From the transconjugants, plasmid DNA was extracted, and the DNA sequences adjacent to *qepA* were determined with primers, starting from both sides of the *qepA*. The minimum inhibitory concentrations (MICs) for donors, transconjugants, and recipient were also measured with MICs of quinolone and several β-lactam antibiotics (Table 1).

PCR amplification, gene mapping, restriction with *HindIII*, and southern hybridization of *HindIII* digestion with *qepA* probe specific

To study the plasmids carrying *qepA* and surrounding genes from five isolates, DNA was extracted from the plasmids

(Axygen kit, California, USA) and separated by 0.6% agarose gel electrophoresis (60 V, 90 minutes) (Promega, Madison, USA). The gels were cut and plasmid DNA fragments of different sizes were extracted. These plasmid DNA fragments served as templates for PCR amplification of *qepA* and surrounding genes, then sequencing, where the positions of these gene plasmids were determined. The size of the plasmid DNAs was estimated.⁸ The amplified products were also purified and digested with *HindIII*. The resulting fragments were ligated into *HindIII*-digested plasmid pBKMV, and transformed into *E. coli* DH10B, which was used for determining quinolone resistance genes. Meanwhile, the resulting fragments were electrophoresed separately through 0.6% agarose gel, and were transferred onto a positively charged nylon membrane (Boehringer, Mannheim, Germany) with capillary transfer. Then, they were subjected to southern hybridization of *HindIII* digestion with *qepA* probe specific. The digoxigenin DNA labeling and detection kits (Boehringer Mannheim) were utilized.

Results

Susceptibility testing

The MICs of antimicrobial agents such as ciprofloxacin, levofloxacin, ofloxacin, nalidixic acid, and others (Table 1) were determined by the dilution methods according to the Clinical and Laboratory Standards Institute guidelines. MIC values of 83 isolates (39 *E. coli*, 29 *E. aerogenes*, and 15 *E. cloacae*) showed ciprofloxacin ≥ 0.25 µg/mL, levofloxacin ≥ 0.25 µg/mL, ofloxacin ≥ 0.5 µg/mL, and nalidixic acid ≥ 16 µg/mL; MIC values of four isolates of *E. coli* (EC2468, EC2751, EC3762, and EC3319) and one isolate of *E. aerogenes* (EA3201) are listed in Table 1. The susceptibility testing results showed that MICs of quinolones, several β-lactam antibiotics, and amikacin for five isolates and the transconjugants were generally much higher than those of *E. coli* J53 (Table 1). Especially, MICs of the transconjugant from isolate EA3201 (EA-ECJ53) at ciprofloxacin, levofloxacin, and ofloxacin are much higher than those of the transconjugants from isolates EC2468, EC2751, EC3762, and EC3319 (EC-ECJ53) (Table 1). Simultaneously, the ratios of

Table 1 Minimum inhibitory concentrations (MICs) of antimicrobial agent for donors, transconjugants, and recipients (µg/mL).

| Antimicrobials | Donors | | Transconjugants | | Recipients |
|----------------|-------------------------|--------|-----------------------|----------|--------------------|
| | EC2468/2751/3762/3319 | EA3201 | EC-ECJ53 | EA-ECJ53 | <i>E. coli</i> J53 |
| Ciprofloxacin | 1/0.5/1/1 | 2 | 0.5/0.5/0.5/0.5 | 1 | 0.0125 |
| Levofloxacin | 1/1/1/1 | 2 | 0.5/0.5/0.25/1 | 1 | 0.0125 |
| Ofloxacin | 2/2/2/2 | 4 | 1/1/1/0.5 | 4 | 0.025 |
| Nalidixic acid | 64/32/64/64 | 64 | 16/16/16/32 | 16 | 4 |
| Piperacillin | 256/128/256/128 | 128 | 64/32/64/32 | 32 | 1 |
| Cefazolin | 256/128/256/256 | 256 | 8/16/4/4 | 4 | 1 |
| Cefuroxime | 128/64/32/32 | 32 | 32/16/16/8 | 8 | 4 |
| Ceftazidime | 2/4/2/1 | 2 | 0.5/1/0.5/0.25 | 0.25 | 0.25 |
| Cefepime | 1/0.5/0.25/0.5 | 0.05 | 0.25/0.125/0.05/0.125 | 0.05 | 0.025 |
| Amikacin | 2,048/4,096/1,024/2,048 | 2,048 | 1,024/2,048/512/1,024 | 1,024 | 0.50 |

EA-ECJ53, *Escherichia coli* transconjugant from isolates EA3201 and *E. coli* J53; EC-ECJ53, *E. coli* transconjugant from isolates EC2468, EC2751, EC3762, EC3319, and *E. coli* J53.

MICs for the transconjugant and recipient isolate *E. coli* J53 at ciprofloxacin, levofloxacin, and ofloxacin are also much higher in 80-fold, 80-fold, and 160-fold levels, respectively.

Genetic structure for *qepA* and surrounding genes

With PCR amplification and sequencing using primers, we sequenced the plasmids of five isolates in lengths. The *qepA3* variant located on ca. 102 kb-length plasmid EA3201, and other *qepA*s located on ca. 124 kb-, 145 kb-, 131 kb-, and 111 kb-length plasmids from isolates EC2468, EC2751, EC3762, and EC3319, respectively. By PCR amplification, the recombinant plasmids were determined to have no other quinolone resistant genes. Gene assembling and analyzing showed that the *qepA3*, *qepA1*, and *qepA2* determinants from isolates EA3201, EC2468, EC2751, EC3762, and EC3319 were flanked or linked by full- or partial-length *tnpA*, *intI1*, *rmtB*, and *bla*_{TEM-1} genes (Figure 1); there were five structures A, B, C, D, and E in these isolates.

Meanwhile, the restriction sites with *Hind*III digestion at *aagctt* are shown in Figure 1 and achieved recombinant plasmids pEA3201, pEC2468, pEC2751, pEC3762, and pEC3319 for ca. 6.2 kb-length plasmid fragments harboring *qepA* and surrounding genes. Southern hybridization of *Hind*III digestion with *qepA* probe specific indicated that these *qepA*s also located on ca. 6.2 kb-length recombinant plasmids.

Putative amino acid variations for *qepA* genes in the isolates

Fourteen-transmembrane (TMS) motifs were analyzed. Three mutations of amino acids in the QepA3 located on 8-, 10- and 12-TMS motifs, but two mutations were out of TMS motifs. The putative amino acid point mutations might be associated with the quinolone resistance in the isolates. Five amino acid mutations were also observed for the novel *qepA3* variant from isolate EA3201, and they were different

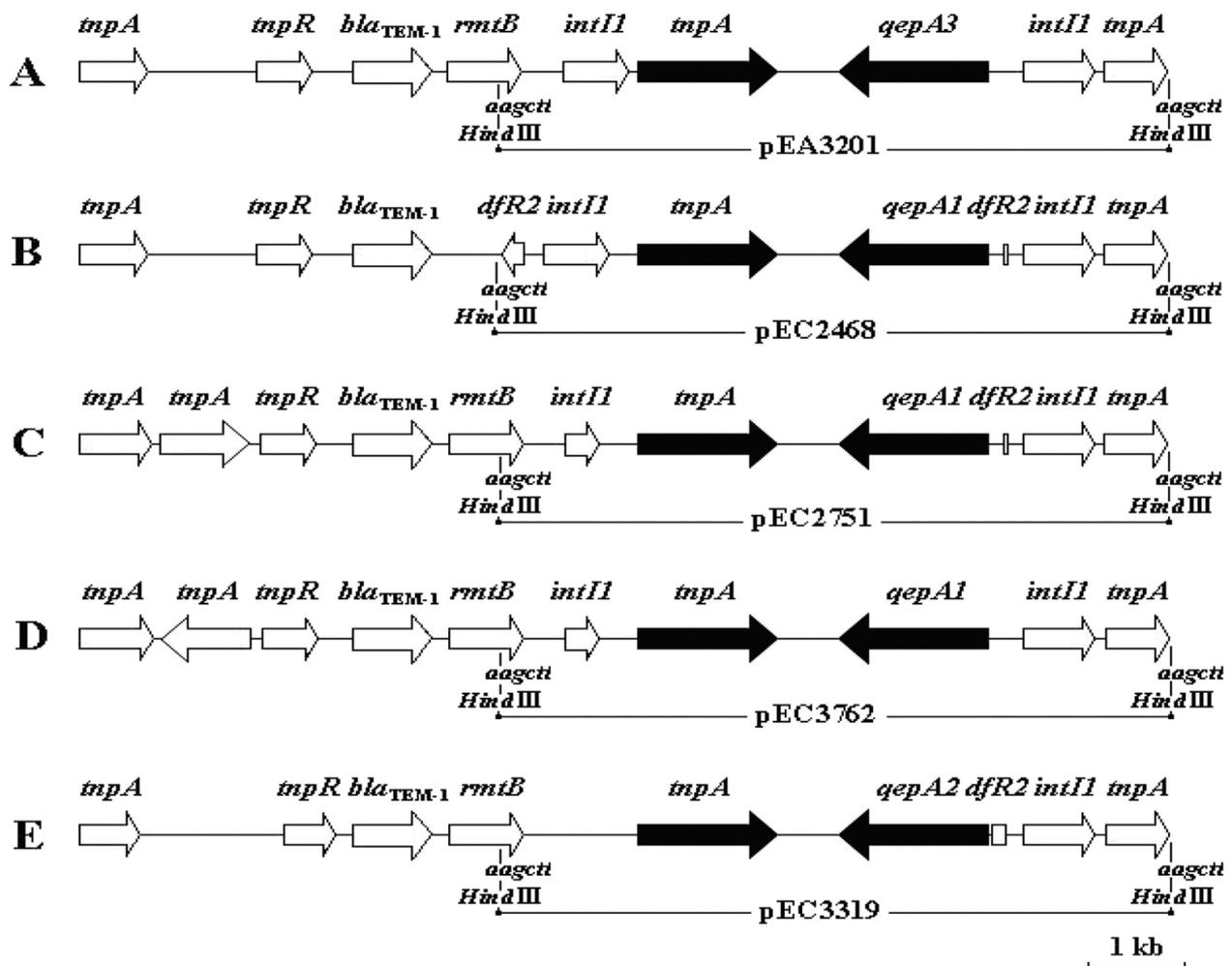


Figure 1. Partial genetic environments of the *qepA3*, *qepA1*, and *qepA2* determinants for plasmids EA3201, EC2468, EC2751, EC3762, and EC3319. The reading frames are shown with the arrowhead indicating the direction of transcription. It identified structures for the black arrows with opposite direction representing *qepA* and *tnpA* genes (ISCR3C transposon determinant). Restriction sites at *aagctt* with *Hind*III are indicated, pEA3201, pEC2468, pEC2751, pEC3762, and EC3319 are recombinant plasmids. (A) the genetic structure A harboring a novel *qepA3* variant gene; (B), (C), and (D), the genetic structure B, C, and D, respectively, harbouring *qepA1* genes; (E) the genetic structure E harboring *qepA2* gene.

at sites Ala (A) 235 Glu (E), Pro (P) 274 Leu (L), Trp (W) 318 Cys (C), Met (M) 372 Lys (K), and Ala (A) 445 Thr (T), when compared with the *qepA1* genes from isolates EC2468, EC2751, and EC3762; the novel *qepA3* variant gene was deposited in GenBank as accession number JQ064560.

Promoter expression for *bla*_{TEM-1}, *rmtB* and novel *qepA3* gene in genetic structure A

There are six genes in the 8663 bp PCR amplicon of genetic structure A, including *bla*_{TEM-1}, *rmtB*, *int11*, novel *qepA3*, and different-length *tnpA* genes. The sites of transcriptional promoter for *bla*_{TEM-1}, *rmtB*, and novel *qepA3* genes are indicated as +1, also shown as -10 and -35 regions.

Discussion

This study determined the lower incidences (5/89) of *qepA* gene among 89 nonrepetitive *E. coli*, *E. aerogenes*, and *E. cloacae* of patient origins and characterized the positive isolates. The presence of *qepA* genes was investigated, and showed characteristics of *qepA* genes in the five isolates.

By analysis of susceptibility testing, the ratios of MICs for the transconjugant from isolate EA3201 and recipient isolate *E. coli* J53 at ciprofloxacin, levofloxacin, and ofloxacin were highest in 80-fold, 80-fold, and 160-fold levels, respectively, much higher than those for the transconjugants from isolates EC2468, EC2751, EC3762, and EC3319 and recipient isolate *E. coli* J53. The ratios were also far higher than those of a reference report.⁸ We guessed it might be relevant to genetic mutation, especially genetic mutation on 8-, 10- and 12-TMS motifs. Therefore, we confirmed the novel *qepA3* could increase the MICs of quinolones at much higher levels; these results also reminded us of the *qepA* dissemination mediating with plasmids.

We determined a novel *qepA* variant from isolate EA3201 located on an approximately 102-kb-long plasmid, and other *qepAs* located on approximately 124-kb-, 145-kb-, 131-kb-, and 111-kb-long plasmids from four other isolates. These *qepA* genes were flanked or linked with full- or partial-length *tnpA*, *int11*, *rmtB*, and *bla*_{TEM-1} genes, and five different genetic structures A, B, C, D, and E were discovered in isolates EA2301, EC2468, EC2751, EC3762, and EC3319, respectively, by gene mapping (Figure 1). However, the common structures were identical with *qepA* and *tnpA* genes (ISCR3C transposon determinants) showing opposite direction in the five structures (Figure 1). Except for the *qepA1* from isolate EC2468 in structure B, the novel *qepA3* and the other *qepA* genes (*qepA1* and *qepA2* determinants) were linked with *rmtB* genes as well in structures A, C, D, and E (Figure 1). The isolate EA3201 was first isolated from a patient's sputum in an intensive care unit ward, and was subsequently discovered in the patient's blood as well. The patient was a farmer, and was once wounded by a farm implement in the field before coming to be hospitalized. We also determined the identical *qepA3* variant from soil bacteria. The isolates EC2751, EC3762, and EC3319 were also from farmers, but we had no evidence to determine if the *qepA* genes came from soil or poultry and livestock. The findings supported a hypothesis

that the uptake of *qepA* from soil bacteria was by *rmtB*-producing *E. coli* or *E. aerogenes*. Meanwhile, A *qepA* was also found in an *rmtB* producing *E. coli* strain isolated in a Belgian hospital, suggesting a probable worldwide dissemination of *qepA* accompanied by *rmtB*, which had been identified in Asia and Europe.^{9,10} Hence, active surveillance of *qepA*-harboring Gram-negative bacteria in soil or animals might reveal a greater prevalence of such kinds of multidrug-resistant microbes.

Conflicts of interest

All authors declare no conflicts of interest.

Acknowledgments

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