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Short Communication

Genome and plasmid context of two *rmtG*-carrying *Enterobacter hormaechei* isolated from urinary tract infections in Brazil

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ABSTRACT

Objectives: Enterobacter hormaechei is an important causative agent of severe infections in critically ill patients. Aminoglycosides are among the main antibiotics for the treatment of E. hormaechei infections, however the development of antimicrobial resistance is an increasing problem. RmtG is a 16S rRNA methyltransferase, a class of enzymes conferring high-level resistance to clinically relevant aminoglycosides. The aim of this study was to characterise the full genetic context of plasmids harbouring the *rmtG* gene in two aminoglycoside-resistant E. hormaechei isolated in Brazil.
Methods: ThermtG-harbouring plasmids were transferred to an *Escherichia coli* J53 recipient strain and were fully sequenced using a MiSeq sequencing system. Complete genome assemblies were accomplished using a combination of Newbler v.3.0, SPAdes 3.10.0 and phrap/cross_match programs. Plasmid sequences were annotated using RAST server and were then manually curated using BLAST databases and ISfinder. Easyfig 2.0 was used to map and compare regions of interest containing *rmtG* in both plasmids.
Results: Both isolates carried the*rmtG* gene on an IncA/C plasmid of ~152 kb and ~235 kb, respectively, associated with a Tn*3* transposon. The plasmids contain a transfer region as well as genes involved in plasmid stability and resistance to β-lactams, sulfonamides and quaternary ammonium compounds. One of the plasmids also carried the *mrk* operon encoding type 3 fimbriae.
Conclusion: This first detection of*rmtG* in *E.* hormaechei supports the ability for horizontal transfer. The location in complex genetic platforms carried by Tn*3* transposons in IncA/C plasmids may facilitate dissemination to other Gram-negative pathogens, further limiting treatment options.

1. Introduction

Enterobacter is a genus of Gram-negative, non-spore-forming bacteria of the family Enterobacteriaceae that is widely distributed in the environment and is also part of the commensal microbiota of the human gut [1]. Most *Enterobacter* clinical isolates belong to the *Enterobacter cloacae* complex (ECC) [2], a heterogeneous group divided into 12 genetic clusters (I–XII) and one sequence crowd (*xiii*) [3]. In recent decades, ECC isolates have emerged as important agents of infections in critically ill patients [2]. In this regard, the intrinsic resistance to some antibiotics and the high ability to upregulate or acquire antimicrobial resistance determinants elevates the clinical significance of these bacteria, which pose a great challenge to public health and to the management of the spread of infections [4]. Identification methods available in most clinical laboratories, such as biochemical tests or mass spectrometry, present poor ability to discriminate members of the

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ECC. However, studies based on molecular characterisation have revealed that *Enterobacter hormaechei* accounts for most clinical isolates [5].

Aminoglycosides are often used in combination with β-lactams to treat severe infections by Gram-negative bacteria. However, the development of various resistance mechanisms against this class of antibiotic is an increasing problem. In the past decade, 16S rRNA methyltransferases (16S RMTases) have emerged globally. These enzymes confer high-level resistance to clinically relevant aminoglycosides, such as amikacin, gentamicin and tobramycin, by catalysing the process of post-transcriptional methylation of the A site of 16S ribosomal RNA (rRNA). Genes encoding acquired 16S RMTases are usually associated with mobile genetic elements carried by plasmids belonging to diverse incompatibility (Inc) groups, which play an important role in the spread of such resistance mechanisms among Gram-negative pathogens [6]. The simultaneous presence of a 16S RMTase gene with other resistance determinants allows the development of multidrug resistance [7]. The RmtG 16S RMTase was first identified in Brazil in *Klebsiella pneumoniae* clinical isolates [8]. Here we report the draft genome of two genetically distinct *E. hormaechei* isolates and the full genetic context of the *rmtG* genes carried by these strains.

2. Materials and methods

2.1. Bacterial isolates and patients

Isolates Ec9 and Ec13 were recovered in 2013 during a study to characterise the diversity of multidrug-resistant *E. cloacae* associated with infections in a tertiary-care hospital located in the State of São Paulo, Brazil. Ec9 was isolated from a urine sample of a patient admitted for sigmoid resection due to perforated diverticulitis. Ec13 was also isolated from a urine sample of a patient admitted for radical nephrectomy due to kidney neoplasia. Both patients were treated for urinary tract infection with meropenem (500 mg/10 mL intravenously).

2.2. Bacterial identification and antimicrobial susceptibility testing

Identification of isolates was initially performed using a VITEK®2 Compact System (bioMérieux, Marcy-l'Étoile, France) as a routine of the microbiology set of the hospital, and also by whole-genome sequencing (WGS) (discussed below) and subsequent identification by average nucleotide identity (ANI) test after submission to the GenBank database.

The antimicrobial susceptibility profile was determined using a VITEK®2 Compact System. Minimum inhibitory concentrations (MICs) for amikacin and gentamicin were also determined using M.I.C.Evaluator™ (M.I.C.E.™) strips (Thermo Fisher Scientific, Basingstoke, UK). The results were interpreted according to Clinical and Laboratory Standards Institute (CLSI) guidelines [9].

2.3. Genome sequence of isolates

Total genomic DNA of strains Ec9 and Ec13 was extracted to construct a Nextera XT DNA library, which was sequenced using a NextSeq platform (Illumina Inc., San Diego, CA, USA). De novo assembly was performed using the Velvet v.1.2.1 pipeline and Geneious R9 (Biomatters Ltd., Auckland, New Zealand). The genome was annotated using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) v.3.2. Multilocus sequence typing (MLST) as well as identification of antimicrobial resistance genes and plasmid replicons were performed using MLST 1.8, ResFinder 2.1 and PlasmidFinder 1.3 databases, respectively, available from the Center for Genomic Epidemiology (http://www.genomicepidemiology.org/).

Fastq files from sequenced genomes were also processed using the BioNumerics calculation engine and the wgMLST plug-in tool (Applied Maths, Sint-Martens-Latem, Belgium). Assembly-free allele detection analyses were performed for each isolate. The wgMLST (15 606 loci synchronised with the in-house schema) dendrogram was produced using categorical differences with 100 scaling factor and complete linkage cluster analysis.

2.4. Next-generation sequencing, assembly and annotation

For determination of the full genetic context of the *rmtG* genes, plasmids from Ec9 and Ec13 (pEc09 and pEc13, respectively) were transferred by broth mating to sodium azide-resistant *Escherichia coli* J53 recipient strain using a previously described protocol, with modifications [10]. Briefly, suspensions of overnight cultures were prepared, with the recipient at one-tenth of the cell density of the donor. Approximately 10^8 CFU of the donor and 10^7 CFU of the recipient were then added to 1 mL of Luria–Bertani broth and were left overnight at 25 °C. The suspension was then diluted in sterile 0.9% saline (10^{-3} and 10^{-5}), was plated onto MacConkey agar containing sodium azide (150 mg/L) and amikacin (100 mg/L), and was incubated overnight at 37 °C to recover *rmtG*-positive transconjugants. Putative transconjugants were confirmed by detection of the *rmtG* gene by PCR as previously described [8]. Plasmid DNA was extracted using a QIAamp® DNA Mini Kit (QIAGEN, Valencia, CA, USA) following the manufacturer's instructions.

Sequencing libraries were constructed using an Illumina TruSeq DNA PCR-Nano Sample Preparation Kit (Illumina Inc.) with a median insert size of 350 bp according to the manufacturer's instructions. Briefly, 100 ng of each plasmid DNA was fragmented using an M220™ Focused-ultrasonicator (Covaris, Brighton, UK) and was end-repaired, A-tailed and adapter-ligated. Library quality control was performed using a 2100 Bioanalyzer System with Agilent High Sensitivity DNA Kit (Agilent Technologies Inc., Santa Clara, CA, USA). Libraries were individually quantified via quantitative PCR (qPCR) using KAPA Library Quantification Kits for Illumina platforms and were pooled together in equimolar quantities and were sequenced on a MiSeq sequencing system (Illumina Inc.) to obtain 2×250-bp paired-end reads.

Complete genome assembly of pEc09 and pEc13 was accomplished using a combination of Newbler v.3.0 (Roche Inc., Basel, Switzerland), SPAdes 3.10.0 [11] and phrap/cross_match (http://www.phrap.org/phredphrapconsed.html) programs. The cross_match tool was used to align the contigs/scaffolds of each assembly against the other. The phrap with consensus sequences from SPAdes assembly was used to close gaps between contigs and scaffolds generated by Newbler assembly until the consensus sequence of each plasmid was obtained.

Plasmids sequences were annotated using the Rapid Annotation using Subsystem Technology (RAST) server (http://rast.nmpdr.org) and were then further curated manually using BLAST databases (http://blast.ncbi.nlm.nih.gov/blast) and ISfinder (http://www-is.biotoul.fr/blast.php). Easyfig. 2.0 was used to map and compare regions of interest containing *rmtG* in both plasmids.

2.5. Nucleotide sequence accession nos

The draft genome sequences of Ec9 and Ec13 have been deposited at DDBJ/ENA/GenBank under accession nos. MUDF00000000 and MUDC00000000, respectively. The versions described in this paper are MUDF01000000 and MUDC01000000, respectively. The plasmid sequences reported in this work have also been deposited to the DDBJ/ENA/GenBank under accession nos. MH325468 and MH325469.

3. Results

Isolates Ec9 and Ec13 were both initially identified as *E. cloacae* by the VITEK®2 Compact System and were then rectified to *E. hormaechei* by the ANI test. Regarding the pattern of antimicrobial susceptibility, Ec9 and Ec13 presented resistance to ertapenem, ceftazidime, ceftriaxone, cefepime, ciprofloxacin and norfloxacin, and susceptibility to imipenem, meropenem and colistin. According to results of the antimicrobial susceptibility test performed by the VITEK®2 Compact System, both strains presented resistance to amikacin (MIC ≥ 64 μg/mL) and intermediate resistance to gentamicin (MIC = 8 μg/mL). However, the MICs determined by M.I.C.E.™ for Ec9 and Ec13 were ≥256 μg/mL for amikacin and ≥1024 μg/mL for gentamicin.

From the 15 606 loci analysed by wgMLST, Ec9 and Ec13 presented 2403 different alleles. Sequencing of Ec9 produced 13 896 066 paired-end reads with a 416 × total coverage. A total of 86 contigs were generated with a G + C content of 55.2% and a 5.3 Mb genome size. Annotation resulted in 5069 coding genes, 54 RNA-encoding genes (49 tRNAs and 5 ncRNAs) and 197 pseudogenes. WGS analysis showed that Ec9 belongs to sequence type 184 (ST184) and carries the aminoglycoside resistance genes *aac(6′)-Ib*, *aadA1* and *rmtG*, the β-lactam resistance genes $bla_{\text{TEM-1a}}$, $bla_{\text{OXA-9}}$, $bla_{\text{ACT-7}}$ and $bla_{\text{CTX-M-2}}$, the fluoroquinolone resistance gene *aac(6′)-Ib*-cr, the fosfomycin resistance gene *fosA*, the MLS_{B} (macrolide–lincosamide–streptogramin B) resistance gene *mph (A)*, the sulfonamide resistance genes *sul1* and *sul2*, and the trimethoprim resistance gene *dfrA1*. Analysis of the genome of Ec9 identified the sequences of two plasmids belonging to the IncFII (Yp) and IncA/C groups.

Sequencing of Ec13 produced 9 902 678 paired-end reads with a 297 × total coverage. A total of 92 contigs were generated with a G + C content of 54.6% and 5.1 Mb genome size. Annotation resulted in 4928 coding genes, 61 RNA-encoding genes (56 tRNAs and 5 ncRNAs) and 207 pseudogenes. WGS analysis showed that Ec13 belongs to ST121 and carries the same resistance genes for aminoglycosides, β-lactams, fluoroquinolones, fosfomycin and sulfonamides as Ec9, differing only by the absence of *mph(A)* and *dfrA1* and the presence of the tetracycline resistance gene *tetC*. Plasmids of incompatibility groups IncA/C, IncFI2A and IncFI2 were identified.

The plasmids carrying *rmtG* in Ec9 (443 × coverage) and Ec13 (143 × coverage) were both identified as belonging to the IncA/C incompatibility group. Sequencing of pEc09 showed 191 predicted genes and a size of 152 054 bp. The core region in pEc09 is composed of the replication initiation protein gene *repA*, the *traABCDEFGHIKLNUVW* genes necessary for plasmid conjugative transfer, and the *parAB* and *stbA* genes involved in plasmid stability. The β-lactamase encoding genes $bla_{\text{OXA-9}}$ and $bla_{\text{TEM-1a}}$, the aminoglycoside resistance genes *aadA1*, *aac(6′)-Ib* and *rmtG*, and the sulfonamide resistance gene *sul2* were identified in a Tn*3* transposon flanked by two copies of the insertion sequence IS*26* (Fig. 1A). Two copies of the *rmtG* gene are associated with *rsmH* and *tgt* genes encoding an rRNA small subunit methyltransferase H (RsmH) and a tRNA-guanine transglycosylase, respectively. The first *rsmH–tgt–rmtG* array is flanked by two copies of a ΔIS*CR2*, and the second *rsmH–tgt–rmtG* array is flanked by one of these copies of ΔIS*CR2* and a complete IS*CR2* (Fig. 2). The presence of this two-fold repeat was confirmed by long-range PCR targeting the region containing the repeat. Analysis of the sequences of *rsmH* in the *rsmH–tgt–rmtG* array of pEc09 showed a substitution g.29764C>T, causing the presence of a stop codon. Since there is a duplication of this region, this mutation also repeats in position 33 776, generating two pseudogenes *rsmH*. The extended-spectrum β-lactamase (ESBL) gene $bla_{\text{CTX-M-2}}$, the quaternary ammonium compound resistance gene *qacEΔ1*, and the sulfonamide resistance gene *sul1* are carried in association with one copy of ISCR*1* (Fig. 1A).

Plasmid pEc13 is 235 699 bp in length and harbours 277 predicted genes. The core region comprises the replication initiation protein gene *repA*, the transfer-related genes *traABCDEFGHIKLMNPQRSUVWXY* and *trbBCEL*, and several genes involved in plasmid stability (*parAB*, *parM*, *stbA*, *psiAB*, *ardA* and *umuCD*) (Fig. 1B). The *rmtG* gene is also present in the *rsmH–tgt–rmtG* array flanked by a ΔIS*CR2* and a complete copy of IS*CR2* (Fig. 2) carried by a Tn*3* transposon along with $bla_{\text{TEM-1a}}$ and *sul2* genes (Fig. 1B). The $bla_{\text{CTX-M-2}}$, *sul1* and *qacEΔ1* genes were also identified in association with IS*CR1* (Fig. 1B).

4. Discussion

As previously discussed, phenotypic methods are unable to distinguish between species of the ECC, which often renders their incomplete identification [2]. In this study, two *E. hormaechei* identified by genomic analysis were initially misidentified as *E. cloacae* by automatic biochemical tests. Correct species identification is clinically relevant as the different clusters of the ECC present different virulence traits [12]. Implementation of sequence-based methods, considered as a reliable tool, is necessary to achieve a more precise identification of these important infections [13].

It was interesting to observe that while the VITEK®2 Compact System identified Ec9 and Ec13 as presenting intermediate resistance to gentamicin, the results obtained by M.I.C.E.™ showed that both strains presented high-level resistance to amikacin and gentamicin, as usually observed for resistance conferred by 16S RMTases [8]. In fact, decreased accuracy of the VITEK®2 automated system in reporting susceptibility to gentamicin was previously reported and, based on this finding, it is suggested that gentamicin should not be considered for the treatment of infections by amikacin-resistant *K. pneumoniae* isolates even if VITEK®2 reports susceptibility to gentamicin [14].

The *rmtG* gene has been mostly described in *K. pneumoniae* [8] and there is also a description in a plasmid of a *Klebsiella aerogenes* [15], both isolated in Brazil. To the best of our knowledge, this is the first report of the presence of *rmtG* in *E. hormaechei*. However, the propensity for recombination and genetic exchange in the ECC is an important feature, and *E. hormaechei* was already discussed as a model of genetic capitalism, a mechanism of accumulation of mobile elements that allows bacteria that already have a selective advantage in a certain setting to adapt even further [16].

The great difference presented by Ec9 and Ec13 by wgMLST shows that the isolates are not related and the acquisition of plasmids containing *rmtG* were independent events. In fact, rapid dissemination of 16S RMTase genes mediated by broad-host-range plasmids such as IncA/C apparently accelerates the acquisition of a multidrug-resistant nature in pathogenic micro-organisms [17].

It is known that 16S RMTase genes are frequently associated with other antimicrobial resistance mechanisms, including CTX-M-type ESBLs [17]. Co-production of RmtG with KPC-2 and an ESBL belonging to the CTX-M group was previously reported in a *K. pneumoniae* isolated in Brazil [8], and co-production of RmtG with CTX-M-2 and CTX-M-15 in a *K. pneumoniae* isolated in the USA [18]. Isolation from humans of *E. cloacae* ST121 and ST184 harbouring different combinations of resistance genes was previously reported [19].

The *rmtG* gene was previously identified in IncA/C plasmids carried by *K. pneumoniae* [18]. The organisational structure of pEc09 suggests that IS*CR2*, in association with IS*26*, also present, is responsible for mobilisation of the array. Furthermore, the association of *rmtG* with IS*CR2* in a two-fold tandem repeat suggests a gene amplification process. In fact, IS*CR2* has been already observed in association with 16S RMTases such as *rmtD* in plasmids and identified as responsible for gene amplifications [6].

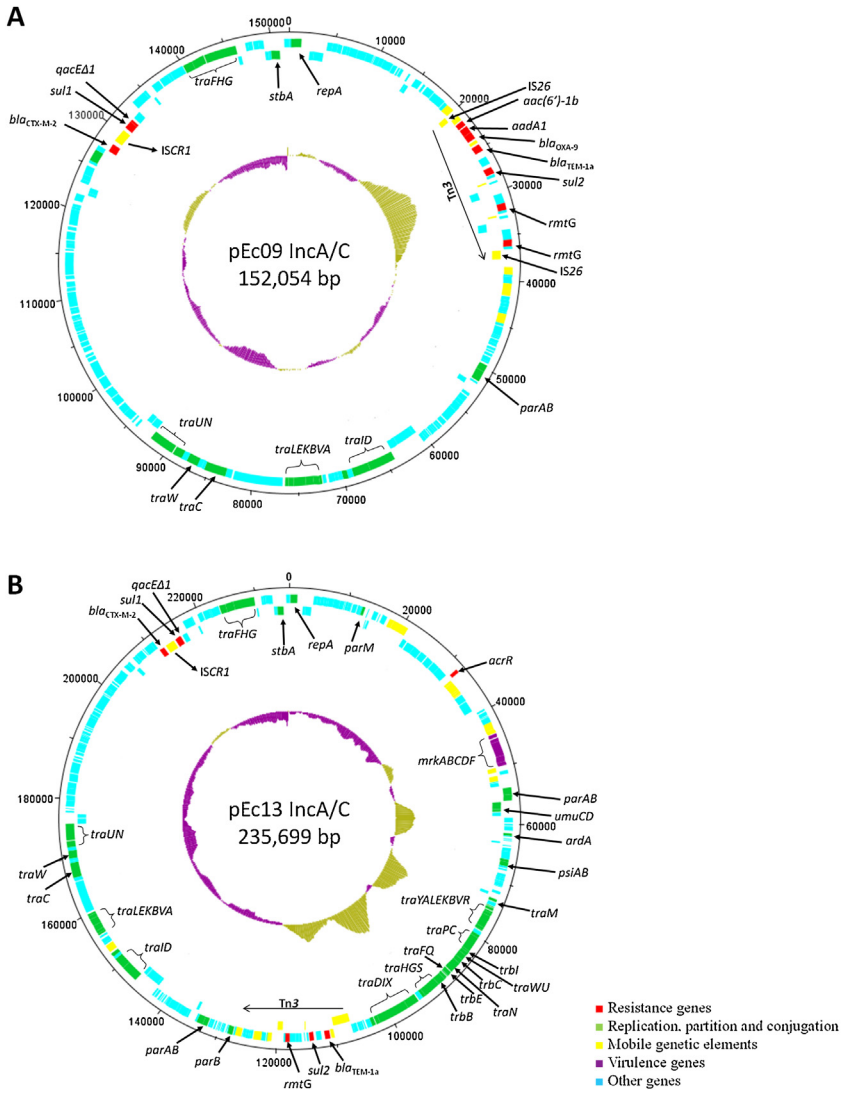

Fig. 1. Structure of IncA/C plasmids (A) pEc09 and (B) pEc13 carrying the *rmtG* gene. The inner circles coloured in magenta and green indicate the G+C content. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

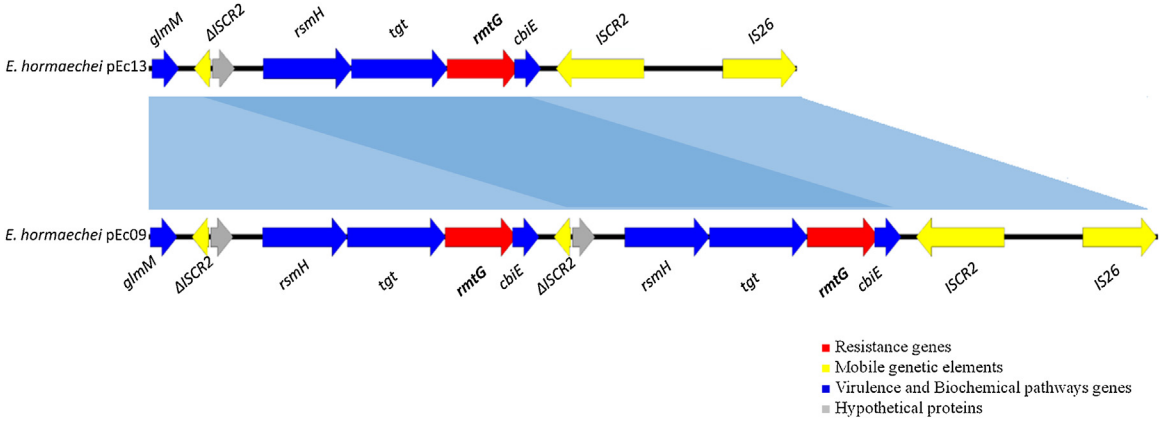

Fig. 2. Comparison of *rmtG*-carrying regions of plasmids pEc09 and pEc13. Light blue shading indicates regions of homology.

None of the patients received any aminoglycoside-based treatment during hospital admission. However, selection of RmtG-producing *K. pneumoniae* in the absence of selective pressure has been reported previously [18].

Plasmid pEc13 also carries the *mrk* operon (*mrkABCDF*) encoding a type 3 fimbriae, produced by various species of Enterobacteriaceae and responsible for adhesion in various components of the extracellular matrix and biofilm formation

[20]. This plasmid-mediated pathogenic feature could be of great importance to the establishment of infection.

5. Conclusions

The genome sequence of RmtG-producing *E. hormaechei* ST184 and ST121 presented here can facilitate comparative genomic analyses among *Enterobacter* strains with distinct antimicrobial susceptibility patterns. The presence of *rmtG* in complex genetic platforms carried by Tn*3* transposons in IncA/C plasmids may allow dissemination of this important mechanism of resistance to other Gram-negative pathogens, further limiting treatment options.

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Competing interests

None declared.

Ethical approval

Ethical approval was obtained from the Ethics Committee of the Faculdade de Medicina de São José do Rio Preto (FAMERP) (São José do Rio Preto, Brazil) [process CEPE 48767315.0.0000.5415].

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