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CITATION:

Gomi, Ryota ...[et al]. Occurrence of class 1 integrons carrying two copies of the blaGES-5 gene in carbapenem-non-susceptible Citrobacter freundii and Raoultella ornithinolytica isolated from wastewater. Journal of Global Antimicrobial Resistance 2021, 26: 230-232

ISSUE DATE:

2021-09

URL:

http://hdl.handle.net/2433/276818

Journal of Global Antimicrobial Resistance 26 (2021) 230–232

Contents lists available at ScienceDirect

Journal of Global Antimicrobial Resistance

journal homepage: www.elsevier.com/locate/jgar

Occurrence of class 1 integrons carrying two copies of the bla_{GES-5} gene in carbapenem-non-susceptible *Citrobacter freundii* and *Raoultella ornithinolytica* isolated from wastewater

Editor: Dr Marisa Haenni

Sir,

Guiana extended-spectrum (GES)-type enzymes are Ambler class A β-lactamases, and some variants, such as GES-5, display carbapenemase activity [1]. Recent studies have reported the occurrence of GES-5-producing Enterobacteriaceae in clinical settings and aquatic environments [2,3]. In our previous study, we detected 38 carbapenemase-producing Enterobacteriaceae (CPE) isolates in wastewater collected in Japan and Taiwan [4]. Genomic characterisation using Illumina sequencing revealed that the bla_{GES-5} gene was the most prevalent carbapenemase-encoding gene among these CPE isolates. The bla_{GES-5} genes were located within two class 1 integrons, In1440 (cassette array, bla_{GES-5}–*aacA31*–*catB8*–*aadA5*) and In1441 (cassette array, bla_{GES-5}–bla_{OXA-17}). However, we could not determine the genetic context of bla_{GES-5} in one *Citrobacter freundii* isolate and one *Raoultella ornithinolytica* isolate owing to the presence of repeat sequences in the genomes. Importantly, short-read assemblies indicated that these two isolates carried multiple bla_{GES-5} genes. Here we characterised two plasmids carrying the bla_{GES-5} genes in these two isolates, respectively.

Citrobacter freundii strain TTHS031 was isolated from a hospital wastewater treatment plant (WWTP) in Tainan City, Taiwan, in September 2015 [4]. *Raoultella ornithinolytica* strain JSWP042 was isolated from a municipal WWTP in the Kansai region of Japan in October 2015 [4]. Both strains were resistant to multiple classes of antibiotics (Supplementary Table S1). De novo hybrid assembly of TTHS031 and JSWP042 using both Illumina and Nanopore reads and genomic analysis were performed as described in the Supplementary Methods.

Hybrid assembly of the genome of *C. freundii* strain TTHS031 revealed the presence of 37 antibiotic resistance genes, including partially deleted genes, in the genome (Supplementary Table S1). Notably, TTHS031 carried one copy of bla_{IMP-8} and three copies of bla_{GES-5}. The bla_{IMP-8} gene was located on a 187 237-bp IncF plasmid and was situated within a previously reported class 1 integron, In73 (cassette array, bla_{IMP-8}–*aacA4*–*catB3*). All of the bla_{GES-5} genes were located on a 149 596-bp plasmid with an IncFII(p14) replicon (Fig. 1a). This plasmid could not be typed by the FAB (FII, FIA, FIB) formula, and no similar plasmids were identified using online BLASTn analysis (all identified hits had <55% query coverage). Hybrid assembly of the genome of *R. ornithinolytica* strain JSWP042 revealed the presence of 15 antibiotic resistance genes in the genome (Supplementary Table S1). Two copies of bla_{GES-5} were located on a 156 755-bp plasmid with IncFII (pBK30683) and IncFIB(K) replicons (Fig. 1b). This plasmid could not be typed by the FAB formula, and online BLASTn analysis did not identify close matches with any other plasmid (all identified hits had <50% query coverage). The bla_{GES-5}-carrying plasmids in TTHS031 and JSWP042 were named pTTHS031_GES and pJSWP042_GES, respectively, and were further characterised as described below. pTTHS031_GES carried two novel class 1 integrons, In1985 and In1986 (Fig. 1c). In1985 contained a gene cassette array of *pgcu180*–bla_{GES-5}–*aacA4*–*gcu79*–*pgcu180*–bla_{GES-5}–*aacA4*–bla_{OXA-1}–*catB3*Δ. The *attC* sites of the first and fifth cassettes of this integron were truncated and thus we refer to these cassettes as *pgcu180*, which indicates pseudo-*gcu180*. The integron was truncated by IS*26*, leading to partial deletion of *catB3*. In1986 contained a gene cassette array of *pgcu180*–bla_{GES-5}–*aacA4*–bla_{OXA-1}–*catB3*. This integron contained a Tn*402*-like *tni* module with insertion of an 8827-bp element in *tniQ*. The inserted element carried inverted repeats (IR) at its ends and a putative transposase gene, which shared 90% nucleotide identity with the transposase gene of IS*Psy42*. Interestingly, the cassette array of In1986 was identical to the partial sequence of In1985 except that *catB3* was truncated in In1985, indicating that both integrons originated from a common ancestor (Supplementary Fig. S1). Both In1985 and In1986 carried a 5′-conserved segment (5′-CS) and contained the weak promoter PcW, which is consistent with the idea that both integrons are related. In1986 was embedded within a Tn*1722*-like putative transposon that carried *tnpR* and *tnpA* of Tn*As1* and *mcp* of Tn*1722*, but no direct repeats were detected on either side of this putative transposon. pTTHS031_GES carried a *tra* region (Fig. 1a), which included genes essential for F transfer (*traIDGEBKLHUAWCFM* and *trbC*) [5]. However, a conjugation attempt using azide-resistant *Escherichia coli* J53 as recipient was unsuccessful. pJSWP042_GES carried one novel class 1 integron, In1987 (Fig. 1d). In1987 carried a gene cassette array of bla_{GES-5}–bla_{GES-5}–$bla_{OXA-932}$–*catB3*–*aadA7*–*aacA4*. This integron contained the 3′-CS (*qacE*Δ*1, sul1, orf5, orf6*), which was followed by an IRt-IS*6100*-IRt element. The 5′-CS was interrupted by an IS*26* element, but the remaining part of the 5′-CS was present adjacent to another IS*26*, which was located 72 855 bp downstream of the IRt-IS*6100*-IRt element. Investigation of the sequences next to the two IS*26* elements revealed the presence of 8-bp target site duplications, namely CATCAGGC and GCCTGATG (a reverse complement of CATCAGGC), which implied that this structure was formed by intramolecular replicative transposition in *trans* of IS*26* [6]. pJSWP042_GES had a highly mosaic structure and carried three different conjugation regions (Fig. 1b). A conjugation attempt using azide-resistant *E. coli* J53 as recipient was unsuccessful, probably due to the truncation of the conjugative regions (see Supplementary Results for details).

Here we characterised two plasmids containing multiple bla_{GES-5} genes. This study revealed the occurrence of Enterobacteriaceae carrying multiple copies of carbapenemase-encoding genes in wastewater, highlighting the need for continuous monitoring of antibiotic resistance in the environment.

https://doi.org/10.1016/j.jgar.2021.06.014

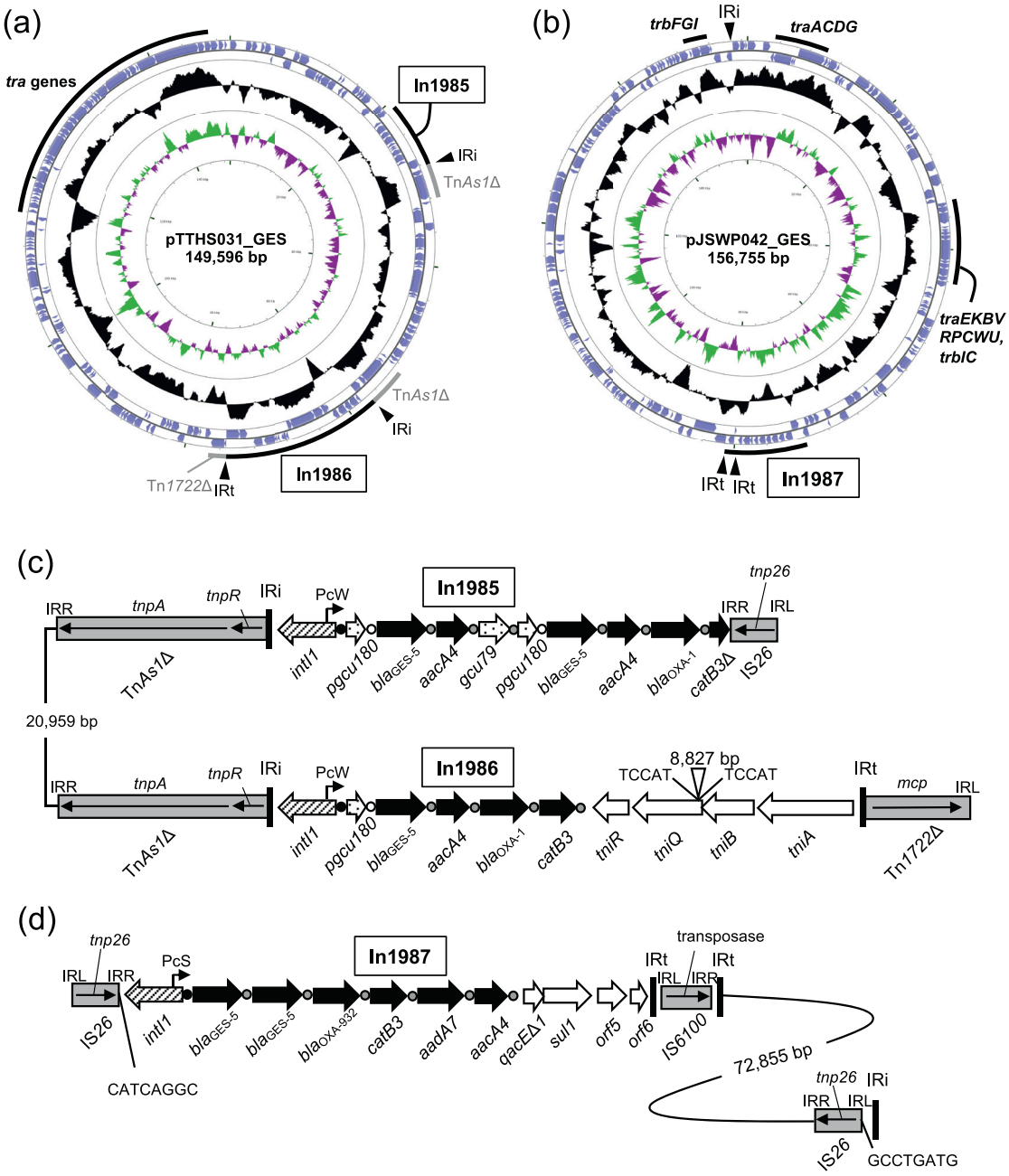

Fig. 1. (a,b) Structure of plasmids pTTHS031_GES (a) and pJSWP042_GES (b). Coding sequences are represented by arrows. The innermost ring shows the GC skew and the middle ring shows the GC content. Images were generated with CGView. (c,d) Schematic representation of the novel class 1 integrons In1985 and In1986 (c) and In1987 (d). Each arrow indicates an encoding gene or a gene cassette. Grey boxes indicate insertion sequences/transposons. Black circles represent *attI* sites, grey circles represent *attC* sites and white circles represent truncated *attC* sites. There is a 20 959-bp segment between Tn*As1*Δ next to In1985 and Tn*As1*Δ next to In1986. *gcu*, gene cassette of unknown function; IRL, inverted repeat left; IRR, inverted repeat right; IRi, inverted repeat at *intI1* end; IRt, inverted repeat at *tni* end.

Nucleotide sequence accession numbers

The sequences of pTTHS031_GES and pJSWP042_GES have been deposited in DDBJ under the accession numbers **LC589514** and **LC589684**, respectively.

Acknowledgment

The authors thank Johann D.D. Pitout for kindly providing *E. coli* strain J53.

Funding

This work was supported by the Japan Society for the Promotion of Science KAKENHI [grant no. JP19K20461].

Competing interests

None declared.

Ethical approval

Not required.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jgar.2021.06.014.

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Revised 6 May 2021