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Genomic landscape of bla_{GES-5}- and bla_{GES-24}-harboring Gram-negative bacteria from hospital wastewater: emergence of class 3 integron-associated bla_{GES-24} genes

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ABSTRACT

Objectives: This study aimed to characterize Gram negative bacteria carrying bla_{GES} carbapenemase genes detected in wastewater from a hospital with no history of detection of clinical isolates producing GES carbapenemases.

Methods: Six hospital effluent samples were screened for carbapenemase-producing organisms (CPO) using CHROMagar mSuperCARBA and MacConkey agar with 1 μg/mL imipenem. Polymerase chain reaction (PCR) amplification and sequencing of carbapenemase genes, multilocus sequence typing, antimicrobial susceptibility testing, and whole-genome sequencing were performed.

Results: Among 21 CPO isolates, 11 *Klebsiella* spp. and 5 *Enterobacter kobei* isolates carried bla_{GES-24}, and 4 *E. roggenkampii* and 1 *Pseudomonas aeruginosa* isolates carried bla_{GES-5}. Genomic analysis of 8 representative isolates comprising 6 bla_{GES-24}-positive and 2 bla_{GES-5}-positive revealed that class 3 integrons with complete or defective Tn*402*-like transposition modules were predominantly associated with two tandem copies of bla_{GES-24}. Furthermore, a total of 5 new class 3 integrons, In3-18 to In3-22, were identified among 5 bla_{GES-24} and 1 bla_{GES-5} plasmids. One strain each of *K. pneumoniae* subsp. *pneumoniae* and *K. quasipneumoniae* subsp. *similipneumoniae* harboring bla_{GES-24} plasmids also carried a rare bla_{VEB-1}-positive class 1 integron on a non-typeable plasmid, where these bla_{VEB-1} plasmids had high sequence similarity. Virulence gene profiles differed between *Klebsiella* spp. and *Enterobacter* spp.; the former harbored type III fimbriae cluster, salmochelin, and T6SS type i2 gene clusters, while the latter had curli pili operon, aerobactin, T2SS gene clusters, and T6SS type i3 gene clusters.

Conclusion: Our findings confirmed the linkage of bla_{GES-24} with rare Tn*402*-like class 3 integrons and the structural diversity of their gene cassette arrays.

1. Introduction

Carbapenemase-producing organisms (CPO) are a global threat, particularly in healthcare settings. Serious limitations in therapeutic options lead to poor clinical outcomes. The major clinically relevant carbapenemases are class A carbapenemase KPC, class B metallo-β-lactamases, IMP, VIM, and NDM, and class D carbapenemase OXA-48. GES carbapenemases remain rare but are increasingly reported worldwide [1]. These carbapenemase-encoding

Abbreviations: KPC, *Klebsiella pneumoniae* carbapenemase; IMP, IMP-type metallo-β-lactamase; VIM, Verona integron-encoded metallo-β-lactamase; NDM, New Delhi metallo-β-lactamase; OXA, Oxacillinase; GES, Guiana extended spectrum β-lactamase.

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genes are frequently associated with mobile genetic elements (MGEs) including transposons, integrons, and plasmids, which promote their mobilization and enable the rapid dissemination of carbapenemase genes [2]. The spread of high-risk clones with flexible ability to uptake, accumulate, and exchange resistance and virulence genes, and the ability to adapt to human hosts and environments, also plays an important role in the successful dissemination of carbapenemase genes [3].

Hospital wastewater is a major reservoir of bacteria with antimicrobial resistance and is a potential hotspot for the horizontal transfer of resistance genes [4]. Higher concentrations of carbapenemase-producing *Enterobacterales* (CPE) isolates in hospital wastewater compared with community wastewater have been noted [5]. Thus, the continuous discharge of hospital wastewater into community sewer drains has raised public health concerns about the potential dissemination of CPE via environmental waterways [6]. Also, there is increasing evidence of CPE cross-transmission between patients and the healthcare water environment, including sink surfaces and wastewater drainage systems, in outbreak-associated cases [7]. Retrograde contamination of the CPO isolates from drainage and wastewater pipe systems have been associated with their transmission from the hospital environment to the patients [8]. Thus, surveillance of hospital wastewater for CPO is important, considering the risk of their retrograde spread from the wastewater drainage system.

In this study, we identified CPO isolates carrying bla_{GES-24} and bla_{GES-5} in wastewater from a hospital with no history of detection of clinical isolates producing GES carbapenemases. Genetic features of plasmids harboring these bla_{GES} genes and their hosts were investigated. Our findings highlight the important role of new Tn*402*-like class 3 integrons in the dissemination of rare bla_{GES-24} genes in hospital wastewater.

2. Material and Methods

2.1. Sample collection and bacterial isolation

Hospital wastewater effluents were collected once per month at two different sampling points from the Nakatsugawa Municipal General Hospital (360-bed capacity) located in Nakatsugawa City, Gifu Prefecture, Japan during the period from June to August 2019. Effluent A was collected at the point where wastewater came from the hospital wards and laboratories, and effluent B was collected at the confluent point of effluent A and the hemodialysis facility wastewater treated in a neutralization tank. All six hospital effluent samples collected in 50-mL sterile tubes were kept under cooling conditions during transportation and were stored at 4°C until analyses.

One mL of well-mixed effluent sample was added to a tube containing 9 mL of brilliant green lactose bile broth (BGLB broth; Eiken Chemical Co., Tokyo, Japan) and incubated overnight at 37 °C with 150 rpm shaking. Then, 10 µL of the broth was plated onto two agar media, MacConkey agar (Eiken) containing 1 µg/mL imipenem (Sigma-Aldrich Japan, Tokyo, Japan) and CHROMagar mSuperCARBA (Kanto Chemical, Tokyo, Japan), followed by overnight incubation at 37 °C. For each effluent sample, representative colonies with different morphological features on agar plates were individually subcultured onto Mueller-Hinton agar (Eiken), which were subjected to matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonics Japan, Yokohama, Japan) using ≥2.000 score cutoffs for species-level identification. The discrimination of *Enterobacter cloacae* complex species was performed by sequencing of two housekeeping genes, *rpoB* and *hsp60*, amplified by polymerase chain reaction (PCR) using specific primers described previously [9,10].

2.2. Molecular analysis

Carbapenemase genes bla_{IMP}, bla_{NDM}, bla_{KPC}, bla_{GES}, and bla_{OXA-48} were screened by PCR and identified by DNA sequencing [11].

Multilocus sequence typing (MLST) analysis was conducted according to the scheme described on the Institute Pasteur MLST website (https://bigsdb.pasteur.fr/) and the PubMLST website (https://pubmlst.org/).

Broth mating conjugation assays were performed with 50 µg/mL ampicillin selection and *Escherichia coli* χ1037 (Rif^r) as the recipient [12]. Transformation assays were performed with NEB 10-beta electrocompetent *E. coli* (DH10B derivative, New England Biolabs, Tokyo, Japan) as the recipient. Transformants were selected on LB agar plates supplemented with ampicillin (50 µg/mL) or aztreonam (16 µg/mL).

2.3. Antimicrobial susceptibility testing

Minimum inhibitory concentrations (MICs) were determined by the broth microdilution method recommended by the Clinical and Laboratory Standards Institute (CLSI) using dry plate DP41 (Eiken Chemical Co., Tokyo, Japan), and the results were interpreted according to CLSI breakpoints [13]. The MICs of faropenem (Sigma-Aldrich), ertapenem (Fujifilm Wako Pure Chemical Co., Osaka, Japan), colistin (Fujifilm), and tigecycline (Tokyo Chemical Industry Co., Tokyo, Japan) were determined by in-house prepared panels according to the CLSI broth microdilution method. *E. coli* ATCC25922 was used as a quality control strain.

2.4. Whole-genome sequencing and data analysis

Illumina sequencing was conducted using the 150-bp paired-end method with the NovaSeq6000 platform (Illumina Inc., San Diego, CA) [12] or partly with the Miseq and the 300-bp paired-end method (Illumina). For MinION (Oxford Nanopore Technologies [ONT], Oxford, UK) sequencing, genomic DNA was extracted using a Qiagen Genomic-tip 20/G kit (Qiagen, Tokyo, Japan). Long-read library preparation was performed using SQK-RBK004 rapid barcoding kit (ONT). Hybrid assembly of Illumina short reads and MinION long reads was performed using Unicycler v0.4.8 [14]. The resulting assemblies were queried with MLST 2.0, ResFinder 4.1, and PlasmidFinder 2.1 available from the Center for Genomic Epidemiology (http://www.genomicepidemiology.org). *Klebsiella* capsule locus (KL) types of *Klebsiella* spp. isolates were assigned using the Kaptive web tool (https://kaptive-web.erc.monash.edu/). Analysis of virulence and heavy metal resistance genes was performed using the VFAnalyzer from the virulence factors database (VFDB, http://www.mgc.ac.cn/VFs/) and BLASTn tool. Detection and subtyping of type VI secretion systems (T6SSs) were conducted using the SecReT6 v3 (https://bioinfo-mml.sjtu.edu.cn/SecReT6/index.php).

For genome-based discrimination of *K. pneumoniae* species complex isolates and *Enterobacter cloacae* complex isolates, ANIm was determined using JspeciesWS (http://jspecies.ribohost.com/jspeciesws/). The following reference genomes were used for ANIm pairwise genome comparisons: *K. pneumoniae* subsp. *pneumoniae* strain ATCC 13883 (GCA_000742135.1), *K. quasipneumoniae* subsp. *quasipneumoniae* strain MGH96 (GCA_001033665.1), *K. quasipneumoniae* subsp. *similipneumoniae* strain ATCC 700603 (GCA_003181175.1), *Enterobacter kobei* strain DSM 13645 (GCA_001729765.1), and *Enterobacter roggenkampii* strain DSM16690 (GCA_001729805.1).

Genome annotation was performed using Prokka on a Galaxy-based platform (Galaxy version 1.14.5), and plasmid comparison was conducted and visualized with EasyFig v2.2.2

Table 1
Twenty-one isolates harboring carbapenemase genes detected from hospital effluents

Bacterial species		Sequence type	Carbapenemase gene	ESBL gene	June, 2019		July, 2019		August, 2019	
MALDI-TOF MS	Molecular techniques[a]				Effluent A	Effluent B	Effluent A	Effluent B	Effluent A	Effluent B
K. pneumoniae species complex	*K. pnueomoniae* subsp. *pneumoniae*	ST12	bla_{GES-24}	bla_{VEB-1}				mS2H5[c]		
	K. quasipneumoniae subsp.	ST4444[b]				IPM1H5[c]	+	+		+
	quasipneumoniae	ST526			mS1O1[c]		+			
	K. quasipneumoniae subsp.	ST1770			mS1O2[c]					
	similipneumoniae	ST4455[b]		bla_{VEB-1}				mS2H7[c]	+	+
E. cloacae complex	*E. kobei*	ST910				IPM1H4[c]	+	+	+	+
	E. roggenkampii	ST963	bla_{GES-5}			IPM1H6[c]	+	+	+	
P. aeruginosa	–	ST3364[b]								IPM3H3[c]

NOTE: The '+' represents that isolates of the same species sharing the same sequence types (STs) were detected.
[a] Species as determined by *hsp60*- and *rpoB*-genotyping for *Enterobacter cloacae* complex and pairwise comparisons of whole-genome sequencing–based average nucleotide identity for *Klebsiella pneumoniae* species complex.
[b] Sequence type newly assigned in this study.
[c] Strains subjected to short- and long-read whole-genome sequencing.

(http://mjsull.github.io/Easyfig/). New sequence types (STs) were assigned by the Institut Pasteur MLST database or the PubMLST database. New integron numbers were assigned by the INTEGRALL database (http://integrall.bio.ua.pt/).

2.5. Phylogenetic analysis of bla$_{GES-5}$- and bla$_{GES-24}$-positive plasmids

The k-mer mash distances among plasmid sequences were calculated using Population Partitioning Using Nucleotide K-mers (PopPUNK) [15] on a Galaxy ARIES-based platform (Galaxy Version 1.1). Construction of a neighbor-joining tree was also performed by PopPUNK by including 45 complete bla_{GES-5}- and bla_{GES-24}-positive plasmids downloaded from the NCBI database (Supplementary Table S1). Evolview v3 (https://www.evolgenius.info/evolview/) was used to visualize the tree and metadata.

3. Results and discussion

3.1. Detection of bacterial isolates harboring carbapenemase genes

For each effluent sample, one representative isolate was chosen from each group sharing the same morphological characteristics on MacConkey agar (1 μg/mL imipenem) or CHROMagar mSuperCARBA and the same bacterial species by MALDI-TOF MS-based identification. In all, 21 isolates comprising *K. pneumoniae* species complex (n = 11), *E. cloacae* complex (n = 9), and *Pseudomonas aeruginosa* (n = 1) were selected for further characterization (Table 1). MLST analysis revealed that the 11 *K. pneumoniae* species complex isolates were assigned to 5 different STs: ST526 (n = 2), ST12 (n = 1), ST1770 (n = 1), ST4444 (a single-locus variant of ST526, n = 4), and ST4455 (n = 3), of which the latter two were newly identified STs. DNA sequencing of the *rpoB* (1,090 bp) and *hsp60* (342 bp) genes and MLST analysis allowed us to assign nine *E. cloacae* complex isolates to *E. kobei* ST910 (five isolates) and *E. roggenkampii* ST963 (four isolates). One *P. aeruginosa* isolate belonged to a new ST, ST3364 (a single-locus variant of ST260).

As summarized in Table 1, ST4444 *K. pneumoniae* species complex isolates, ST910 *E. kobei* isolates, and ST963 *E. roggenkampii* isolates were repeatedly detected from hospital effluents A and/or B through three times of sampling. *Klebsiella pneumoniae* species complex isolates of ST526 and ST4445 were also detected continuously twice from effluents. PCR and Sanger sequencing analysis revealed that the 11 *K. pneumoniae* species complex isolates and 5 *E. kobei* isolates carried the bla_{GES-24} gene, and 4 *E. roggenkampii* isolates and a *P. aeruginosa* isolate carried the bla_{GES-5} gene. Eight representative isolates consisting of the first identified isolates of the same species belonging to the same STs and harboring the same bla_{GES} genes (*K. pneumoniae* species complex strains mS2H5, IPM1H5, mS1O1, mS1O2, mS2H7, *E. kobei* strain IPM1H4, *E. roggenkampii* strain IPM1H6, and *P. aeruginosa* strain IPM3H3) were subjected to further analysis and whole-genome sequencing.

3.2. Genomic characteristics of isolates harboring bla$_{GES-24}$ and bla$_{GES-5}$

The hybrid assembly of Novaseq (or partly Miseq)/MinION sequence reads of the eight representative isolates yielded their complete circular chromosome sequences of 4,864,738 bp to 6,726,163 bp (except for *K. pneumoniae* species complex strain IPM1H5) and 1 to 19 plasmid scaffolds (Table 2).

Five *K. pneumoniae* complex strains were allocated to the species: *K. pneumoniae* subsp. *pneumoniae* strain mS2H5 (99.2% ANIm with *K. pneumoniae* subsp. *pneumoniae* strain ATCC 13883), *K. quasipneumoniae* subsp. *quasipneumoniae* strains IPM1H5 and mS1O1 (99.1% ANIm with *K. quasipneumoniae* subsp. *quasipneumoniae* strain MGH96), and *K. quasipneumoniae* subsp. *similipneumoniae* strains mS1O2 and mS2H7 (99.2% ANIm with *K. quasipneumoniae* subsp. *similipneumoniae* strain ATCC 700603). *Enterobacter kobei* strain IPM1H4 and *E. roggenkampii* strain IPM1H6 produced 99.1% ANIm with *E. kobei* DSM 13645 and 98.6% ANIm with *E. roggenkampii* strain DSM16690, respectively (Tables 1 and 2).

Profiles of antimicrobial resistance genes varied among the eight strains (Table 2). Notably, besides bla_{GES-24}, bla_{VEB-1}, *aacA4*, *aadB*, and *qnrVC4* were found in *K. pneumoniae* subsp. *pneumoniae* mS2H5 and *K. quasipneumoniae* subsp. *similipneumoniae* mS2H7, of which the latter also carried a new $bla_{OXA-921}$ gene. A difference in virulence gene carriage between five *Klebsiella* spp. and two *Enterobacter* spp. was noted (Table 2 and Supplementary Fig. S1); the former harbored type III fimbriae cluster *mrkABCDFHIJ*, salmochelin *iroE*, and T6SS type i2 gene clusters, whereas the latter had curli pili operon *csgADFG* (*E. roggenkampii* IPM1H6 had *csgD* disrupted by integrating IS*5* family transposase), aerobactin *iucABCD-iutA*, type II secretion system (T2SS) gene clusters, and T6SS type i3 gene clusters. In *K. quasipneumoniae* subsp. *similipneumoniae* mS2H7, plasmid-associated *mrkABCDFJ* and chromosome-encoded *mrkHI* were identified [16]. All eight strains had several genetic determinants conferring resistance to heavy metals such as copper, silver, mercury, tellurite, arsenic, and cobalt on their chromosomes and/or plasmids.

Four of five *Klebsiella* spp. strains had outer membrane protein (OMP) genes *ompK36* and/or *ompK35* disrupted; disruption of both *ompK36* (IS*5* insertion) and *ompK35* (IS*1* × *4* insertion) in strain IPM1H5, *ompK35* (IS*1* × *4* insertion) in mS1O1, *ompK35* (nonsense mutation) in mS2H5, and both *ompK36* (threonine-aspartate duplication) and *ompK35* (nonsense mutation) in mS2H7. Also, the dis-

Table 2
Genetic features of representative isolates harboring the bla_{GES-24} and bla_{GES-5} genes

Strains	Species, STs	Chromosome size (bp)	KL type	Antimicrobial resistance genes		Virulence-associated genes		Heavy metal resistance genes		Plasmid Inc types
				Chromosome	Plasmid	Chromosome	Plasmid	Chromosome	Plasmid	
mS1O1	*K. quasipneumoniae* subsp. *quasipneumoniae* ST526	5,178,147	KL28	$bla_{OKP-A-11}$, *fosA*, *oqxA*, *oqxB*	bla_{GES-24} (2 copies), *catB3*	*entBF*, *fepABCDG*, *fes*, *iroE*, *iutA*, *kfuA*, *mrkABCDFHIJ*, *rcsAB*, T6SS-i2 (*ompA*, *tssABCDFGHIJKL*, *vasK*, *vgrG*),T6SS-unknown1(*impA*, *ompA*, *tssEFGJKL*, *vasK*, *vgrG*)	*traT*	-	*terBCDWZ*, *silBCFGRS*, *pcoA*	Col(pHAD28), Col440I, IncFIB(K), IncFIB(Mar), IncFII(K), IncFII(pBK30683)
mS1O2	*K. quasipneumoniae* subsp. *similipneumoniae* ST1770	5,473,547	KL137	$bla_{OKP-B-3}$, *fosA*, *oqxA*, *oqxB*	bla_{GES-24}	*entBF*, *fepABCDG*, *fes*, *iroE*, *iutA*, *kfuB*, *mrkABCDFHIJ*, *rcsAB*, T6SS-i2 (*ompA*, *tssABCDFGHIJKL*, *vasK*, *vgrG*)	*traT*	-	*terBCDEYZ*, *merRTPCADE*	ColpVC, IncFIA(HI1), IncFIB(K), IncFIB(Mar), IncFIB(pQil), IncFII(Yp), IncFII(K), IncFII(pBK30683), IncX5
IPM1H5	*K. quasipneumoniae* subsp. *quasipneumoniae* ST4444	incomplete	KL28	$bla_{OKP-A-11}$, *fosA*, *oqxA*, *oqxB*	bla_{GES-24} (2 copies)	*entBF*, *fepABCDG*, *fes*, *iroE*, *iutA*, *kfuAB*, *mrkABCDFHIJ*, *rcsAB*, T6SS-i2 (*ompA*, *tssABCDFGHIJKL*, *vasK*, *vgrG*), T6SS-unknown1(*impA*, *ompA*, *tssEFGJKL*, *vasK*, *vgrG*)	*traT*	-	*terBCDWZ*, *silBCFGRS*, *pcoA*	Col(pHAD28), Col(Ye4449), Col440I, IncFIB(K), IncFII(K), IncFII(pBK30683)
IPM1H4	*E. kobei* ST910	4,958,727	-	bla_{ACT-87}, *fosA*	bla_{GES-24} (2 copies)	*csgADFG*, *entBF*, *fepABCDG*, *fes*, *iucABCD*, *iutA*, *rcsAB*, T2SS (*ftsY*, *gspCDEFGHKLM*, *secABDEFGMY*, *tatABCE*, *yajC*, *yidC*), T6SS-i2 (*clpK*, *ompA*, *tssBCDHIKL*, *vgrG*), T6SS-i3 (*tssABCDEFGHIJKLM*, *vgrG*)	-	*pcoBCERS*, *silFR*	*arsRBC*	Col(pHAD28), Col(Ye4449), Col440I, ColE10, IncFIB(K), IncFII(pSFO), IncQ3
IPM1H6	*E. roggenkampii* ST963	4,864,738	-	bla_{MIR-10}	bla_{GES-5}, *aacA4*, *sul1*, *qnrS2*	*cfaB*, *csgAFG*, *entBF*, *fepABCDG*, *fes*, *hemCEGHLW*, *iucABCD*, *iutA*, *rcsAB*, T2SS (*ftsY*, *gspCDEFGHKLM*, *secABDEFGMY*, *tatABCE*, *yajC*, *yidC*), T6SS-i3 (*dotU*, *tssABCDEFGHJKLM*, *vgrG*)	-	*cusA*, *terZABCDEF*	*merRTPCADE*, *merRTPFA*, *merDE*	Col440I, IncFIB(pECLA), IncFII(Yp), IncX5, IncQ3
mS2H5	*K. pneumoniae* subsp. *pneumoniae* ST12	5,404,011	KL122	bla_{SHV-11}, *fosA*, *oqxA*, *oqxB*	bla_{GES-24} (4 copies), bla_{VEB-1}, *catB3* (2 copies), *aacA1*, *aacA4*, *aadB*, *qnrVC4*	*entBF*, *fepABCDG*, *fes*, *iroEN*, *iutA*, *mrkABCDFHIJ*, *rcsAB*, *uge*, T6SS-i2 (*ompA*, *tssABCDFGHIJKL*, *vasK*, *vgrG*), T6SS-unknown1(*impA*, *ompA*, *tssEFGJKL*, *vasK*, *vgrG*), T6SS-unknown2(*ompA*, *tssEFGJKL*, *vasK*, *vgrG*)	*traT*, *mrkABCDFJ*	*cusABCF*	*terABCDEXZ*, *merRTPCADE*	Col(pHAD28), Col440I, Col440II, IncFIB(Mar), IncFII(K), IncFII(pBK30683)
mS2H7	*K. quasipneumoniae* subsp. *similipneumoniae* ST4455	5,166,132	KL158	$bla_{OKP-B-49}$, *fosA*, *oqxA*, *oqxB*	bla_{GES-24} (3 copies), bla_{VEB-1}, $bla_{OXA-921}$, *sul1*, *aacA4*, *aadB*, *qnrVC4*, *dfrA14*	*entBF*, *fepABCDG*, *fes*, *iroEN*, *iutA*, *kfuB*, *mrkHI*, *rcsAB*, T6SS-i2 (*ompA*, *tssABCDFGHIJKL*, *vasK*, *vgrG*)	*mrkABCDFJ*		*terABCDEXZ*, *merRTPCADE*	Col(pWES), Col(pHAD28), Col(Ye4449), ColpVC, Col440II, IncFIA(HI1), IncFIB(K), IncFIB(Mar), IncFII(K), IncP6, IncQ2
IPM3H3	*P. aeruginosa* ST3364	6,726,163	-	bla_{PDC-3}, $bla_{OXA-904}$, *aph(3′)-IIb*, *crpP*, *fosA*, *catB7*	bla_{GES-5}, *aacA4*	T1SS (*apr*, *hasAp*, *hlyB*), T2SS (*cbpD*, *lasAB*, *lipAC*, *phoA*, *plcBHN*, *prpL*, *toxA*), T3SS (*exoSTY*), QS (*lasIR*, *rhlIR*), *algD*, *fliC*, *phzHMS*, *pilAB*, *pvdA*, *rhlAB*	-	*copAB*, *czcA*	-	-

entBF, enterobactin synthase; *fepABCDG*, ferri-enterobactin transport operon; *fes*, ferric enterobactin esterase; *iroEN*, salmochelin siderophore system; *iucABCD*, aerobactin synthesis; *iutA*, ferric aerobactin receptor; *kfuAB*, ferrous iron uptake system; *mrkABCDFHIJ*, type III fimbriae cluster; *rcsAB*, regulation of capsule synthesis; *uge*, uridine diphosphate galacturonate 4-epimerase; *traT*, complement resistance protein; *cfaB*, CFA/I fimbriae; *csgADFG*, curli pili operon; *hemCEGHLW*, heme biosynthesis; *ftsY, gspCDEFGHKLM, secABDEFGMY, tatABCE, yajC*, and *yidC*, T2SS-associated proteins; *clpK, dotU, impA, ompA, tssABCDEFGHIJKLM, vasK*, and *vgrG*, T6SS-associated proteins; *apr*, alkaline protease; *hasAp*, heme acquisition protein; *hlyB*, T1SS ATP-binding cassette (ABC) protein; *cbpD*, chitin-binding protein; *lasA*, elastase A; *lasB*, elastase B; *lipA*, lipase A; *lipC*, lipase C; *phoA*, alkaline phosphatase; *plcB*, phospholipase C; *plcH*, hemolytic phospholipase C; *plcN*, non-hemolytic phospholipase C; *prpL*, protease IV; *toxA*, exotoxin A; *exoT*, RhoGAP/ADPR; *exoY*, adenyl cyclase; *exoS*, Rho GTPase-activating protein [RhoGAP]/ADP-ribosyl-transferase [ADPRT]; *lasIR, rhlIR*, quorum-sensing systems; *algD*, GDP-mannose 6-dehydrogenase; *fliC*, flagellar protein; *phzH*, phenazine-modifying enzyme; *phzM*, phenazine-specific methyltransferase; *phzS*, 5-methylphenazine-1-carboxylate 1-monooxygenase; *pilAB*, type IV pilin protein; *pvdA*, ornithine monooxygenase; *rhlAB*, Rhamnosyl transferase operon; *pcoABCERS*, copper resistance proteins; *silBCFGRS*, silver resistance proteins; *cusABCF*, copper/silver efflux pump; *terABCDEFWXYZ*, tellurite resistance proteins; *copA*, copper-exporting P-type ATPase; *copB*, probable copper-transporting P-type ATPase B; *czcA*, cobalt-zinc-cadmium resistance protein; *arsRBC*, arsenic resistance operon; *merRTPFCADE*, mercury resistance proteins.

Table 3
MICs of antimicrobials for eight representative isolates harboring bla_{GES-24}, bla_{GES-5}, and bla_{GES-24}/bla_{VEB-1} genes

Antimicrobial agents	MIC (μg/mL)							
	June, 2019					July, 2019		August, 2019
	Effluent B	Effluent B	Effluent B	Effluent B	Effluent B	Effluent B		Effluent B
	Kqq ST526 mS1O1 (bla_{GES-24})	*Kqs* ST1770 mS1O2 (bla_{GES-24})	*Kqq* ST4444 IPM1H5 (bla_{GES-24})	*Ek* ST910 IPM1H4 (bla_{GES-24})	*Er* ST963 IPM1H6 (bla_{GES-5})	*Kpp* ST12 mS2H5 (bla_{GES-24}, bla_{VEB-1})	*Kqs* ST4455 mS2H7 (bla_{GES-24}, bla_{VEB-1})	*Pa* ST3364 IPM3H3 (bla_{GES-5})
Ampicillin	>16	>16	>16	>16	>16	>16	>16	>16
Piperacillin	64	64	>64	32	32	>64	>64	>64
SAM	>16-8	>16-8	>16-8	>16-8	>16-8	>16-8	>16-8	>16-8
TZP	16-4	32-4	64-4	≤4-4	8-4	16-4	>64-4	>64-4
Cefazolin	>16	>16	>16	>16	>16	>16	>16	>16
Cefotiam	>4	>4	>4	>4	>4	>4	>4	>4
Ceftazidime	4	4	8	2	2	>16	>16	>16
Cefpodoxime	4	4	4	4	>4	>4	>4	>4
Ceftriaxone	≤1	2	4	≤1	≤1	2	16	>32
Cefepime	≤2	≤2	≤2	≤2	≤2	≤2	8	16
Cefmetazole	>32	>32	>32	>32	>32	32	>32	>32
Flomoxef	>16	>16	>16	>16	>16	4	>16	>16
Aztreonam	≤2	≤2	≤2	≤2	≤2	>16	>16	16
Faropenem[a]	32	>64	>64	64	>64	64	>64	>64
Ertapenem[a]	4	16	64	32	8	2	32	>64
Imipenem	4	4	>8	>8	8	4	>8	>8
Meropenem	4	8	>8	>8	>8	0.5	>8	>8
Gentamicin	≤2	≤2	≤2	≤2	≤2	≤2	≤2	>8
Amikacin	16	≤8	≤8	≤8	≤8	16	≤8	16
Minocycline	≤2	≤2	≤2	≤2	≤2	4	≤2	>8
Levofloxacin	0.25	4	0.25	0.5	2	0.5	1	2
Fosfomycin	≤32	>128	≤32	≤32	≤32	128	64	≤32
SXT	≤9.5-0.5	≤9.5-0.5	≤9.5-0.5	≤9.5-0.5	≤9.5-0.5	≤9.5-0.5	>38-2	>38-2
Colistin[a]	0.25	0.5	0.25	0.25	32	0.25	0.25	2
Tigecycline[a]	1	0.5	1	0.5	0.5	0.5	0.5	>4

Kqq, Klebsiella quasipneumoniae subsp. *quasipneumoniae; Kqs, K. quasipneumoniae* subsp. *similipneumoniae; Kpp, K. pneumoniae* subsp. *pneumoniae; Ek, Enterobacter kobei; Er, E. roggenkampii; Kp, K. pneumoniae*; MIC, minimum inhibitory concentration; *Pa, Pseudomonas aeruginosa*; SAM, ampicillin-sulbactam; TZP, piperacillin-tazobactam; SXT, trimethoprim-sulfamethoxazole.

[a] MICs were determined using microdilution panels prepared in-house.

ruption of both *ompC* and *ompF* was detected in two *Enterobacter* spp. strains; disruption of *ompC* (IS*Ppu12* insertion) and *ompF* (nonsense mutation) in strain IPM1H4 and *ompC* (nonsense mutation) and *ompF* (nonsense mutation) in IPM1H6.

3.3. Antimicrobial susceptibility of isolates harboring the bla$_{GES-24}$ and bla$_{GES-5}$ genes

As shown in Table 3, all strains showed resistance to ertapenem (MIC 2->64 μg/mL), imipenem (MIC 4->8 μg/mL), and meropenem (MIC 4->8 μg/mL) except for one strain having a meropenem MIC of 0.5 μg/mL (*K. pneumoniae* subsp. *pneumoniae* mS2H5). Those strains had faropenem MICs of 32 to >64 μg/mL. They were susceptible to amikacin (MIC ≤8-16 μg/mL). Also, *K. pneumoniae* subsp. *pneumoniae* mS2H5 and *K. quasipneumoniae* subsp. *similipneumoniae* mS2H7, both of which co-harbored bla_{GES-24} and bla_{VEB-1} genes, were resistant to ceftazidime (MIC >16 μg/mL) and aztreonam (MIC >16 μg/mL). *E. roggenkampii* IPM1H6, which was confirmed to carry no *mcr* genes, expressed a higher level of resistance to colistin (MIC 32 μg/mL).

The eight strains were analyzed for their ability to transfer ampicillin resistance to recipient *E. coli*. Five of them yielded ampicillin-resistant transconjugants harboring plasmids carrying bla_{GES-24} or bla_{GES-5} with frequencies ranging from 4.9×10^{-7} to 8.3×10^{-6} CFU/donor cells (Table 4). Neither of the two isolates co-harboring bla_{GES-24} and bla_{VEB-1} yielded aztreonam-resistant transconjugants.

3.4. Analysis of bla$_{GES-24}$- and bla$_{GES-5}$-harboring plasmids

Complete circular sequences of six bla_{GES-24}-carrying plasmids, pmS1O1-GES24, pIPM1H5-GES24, pIPM1H4-GES24, pmS2H7-GES24, pmS2H5-GES24_1, and pmS2H5-GES24_2, and two bla_{GES-5}-carrying plasmids, pIPM1H6-GES5 and pIPM3H3-GES5, were obtained. The bla_{GES-24}-carrying plasmid pmS1O2-GES24 harbored by *K. quasipneumoniae* subsp. *similipneumoniae* mS1O2 could not be circularized. These eight circular plasmids varied in size from 18.0 to 129.5 kb, as shown in Table 4. It is of note that pIPM1H5-GES24 and pmS2H5-GES24_1 carried two replicons of Col(pHAD28) and IncFII(pBK30683), while pmS2H7-GES24 carried three replicons of Col(pWES), IncP6, and IncQ2 (Table 4). In five bla_{GES-24}-harboring plasmids, the bla_{GES-24} genes were found to be located on new class 3 integrons, In3-18 (pmS1O1-GES24), In3-19 (pIPM1H5-GES24 and pIPM1H4-GES24), In3-21 (pmS2H5-GES24_1), and In3-22 (pmS1O2-GES24), where the former three contained two tandem copies of bla_{GES-24} (Fig. 1A). The plasmid pmS2H5-GES24_2 also contained two tandem copies of bla_{GES-24} flanked upstream by a truncated *intI3* gene. In the plasmid pmS2H7-GES24, a new class 1 integron, In2067, contained two tandem copies of bla_{GES-24} and IS*Spu7*-associated bla_{GES-24}. The bla_{GES-5} genes were within new class 3 integron, In3-20, and new class 1 integron, In2005, located on non-typeable plasmids pIPM1H6-GES5 and pIPM3H3-GES5, respectively (Fig. 1B). These class 1 and 3 integrons carrying bla_{GES-24} and bla_{GES-5} were associated with Tn*402*-like transposons possessing complete or defective *tniABQR* transposition module. The mercury resistance operon *merRTPCADE* was located on three plas-

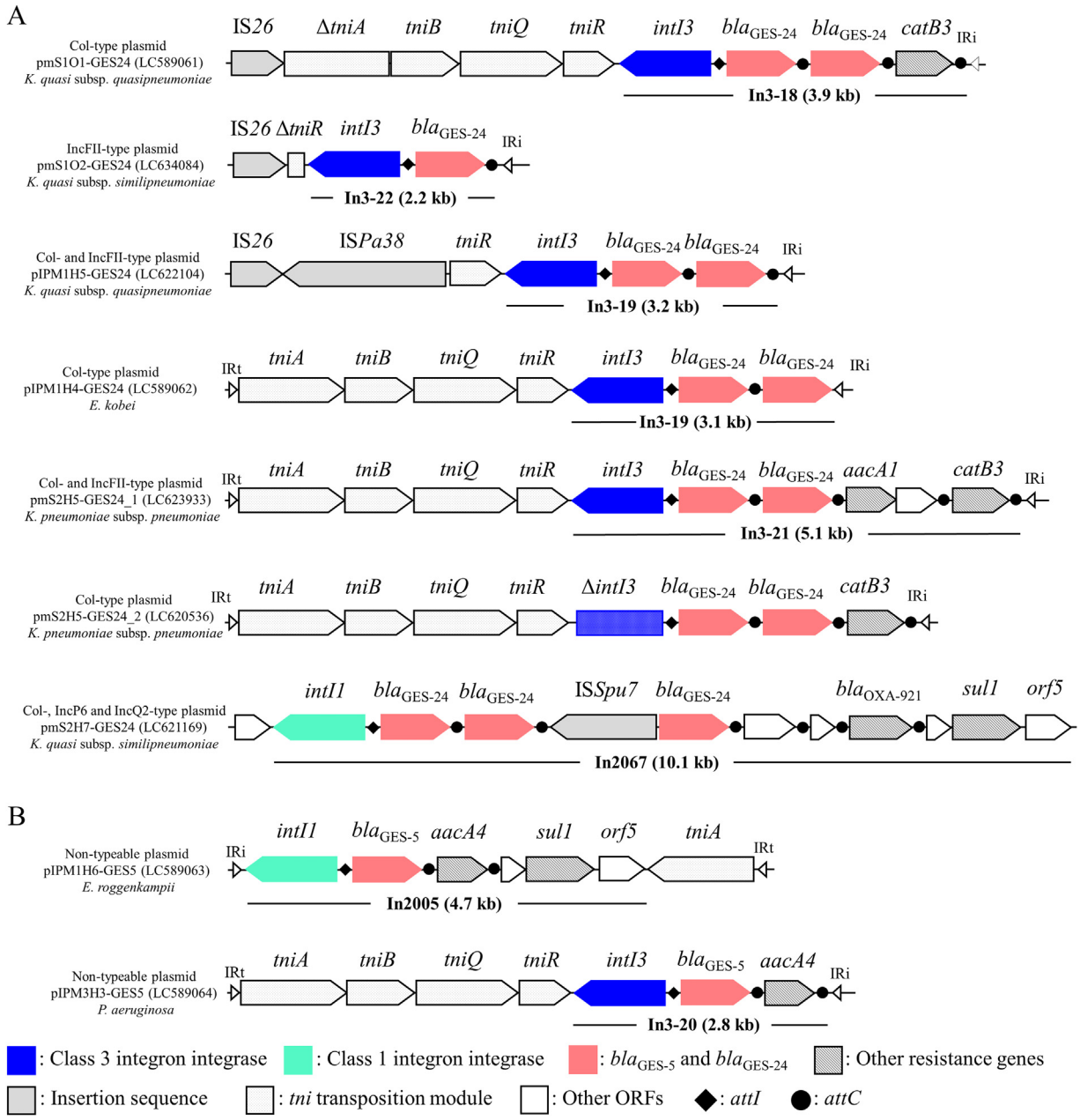

Fig. 1. Schematic representation of regions surrounding class 1 and 3 integrons carrying the bla_{GES-24} and bla_{GES-5} genes described in this study. (A) Structure of the four new class 3 integrons and one new class 1 integron carrying bla_{GES-24}. (B) Structure of the one new class 1 integron and one new class 3 integron carrying bla_{GES-5}.

mids, pmS1O2-GES24, pmS2H5-GES24_1, and pmS2H7-GES24. It was found that a 3,336-bp region containing the klebicin B structural gene and klebicin B immunity gene, flanked by 5-bp direct repeats (TAAAGG) shared by three plasmids, pmS2H5-GES24_1, pIPM1H5-GES24, and pIPM1H4-GES24, exhibited 99.8% nucleotide sequence identity (100% query coverage) to that of our previously described plasmid, pCol440I (GenBank LC505603), harbored by *K. pneumoniae* A1-1 strain detected from municipal wastewater influent in Japan [17].

The transformants that had gained bla_{GES-24} or bla_{GES-5} genes showed higher MICs to cefmetazole (MIC 32->32 μg/mL), flomoxef (MIC 8-16 μg/mL), and faropenem (MIC 16->64 μg/mL) than those for *E. colli* NEB10-beta recipient strain, while they showed slightly increased MICs to ceftazidime (MIC 2-4 μg/mL) and imipenem (MIC 0.5-2 μg/mL), as shown in Supplementary Table S2.

3.5. Population structure of bla_{GES-5} and bla_{GES-24} plasmids

A neighbor-joining tree of the k-mer distances of the whole genome of 54 plasmids and their metadata is shown in Fig. 2. Six bla_{GES-24} plasmids of Col(pHAD28), IncFII(pBK30683), and Col(pHAD28)/IncFII(pBK30683), and one non-typeable bla_{GES-5} plasmid in this study, all of which harbored class 3 integron-associated bla_{GES} genes, were clustered together. These plasmids were genetically related to plasmids carrying bla_{GES-5} and bla_{GES-24} recovered from *Enterobacterales* isolates of human and sewage origins. Interestingly, the clade that consisted of most plasmids in this study was located separately from two distinct clades, one consisting mainly of IncC plasmids harboring class 1 integron-associated bla_{GES-24} genes from hospital sewage in Japan, and the other consisting mainly of IncF plasmids harboring class 1 integron-

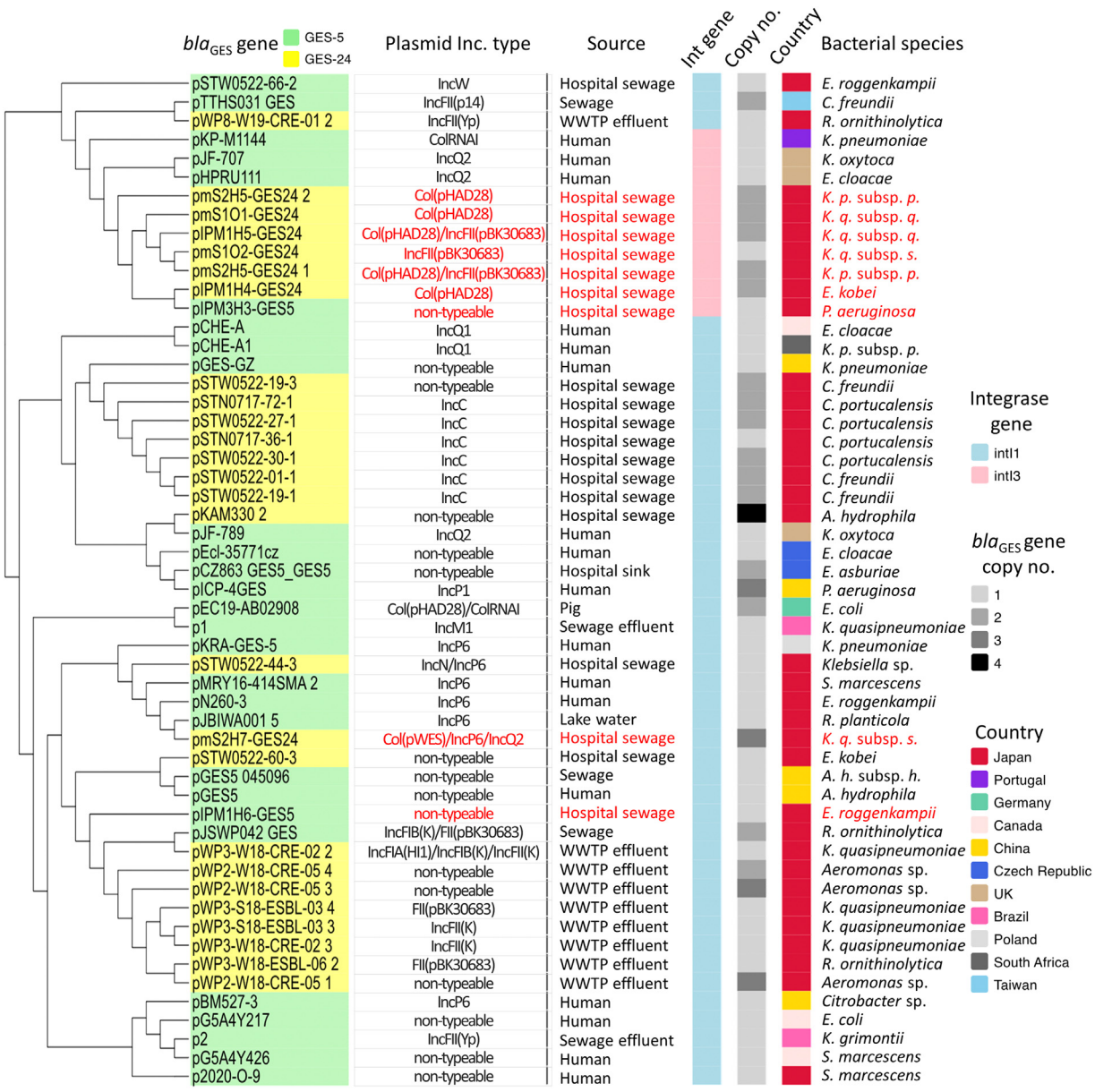

Fig. 2. Phylogenetic analysis of 54 plasmids harboring bla_{GES-24} and bla_{GES-5} of Gram-negative bacteria isolated from various sources. Inc types, sources, and bacterial species are indicated at the right of the tree, with data on nine plasmids in this study marked in red. Integrase genes, bla_{GES} gene copy numbers, and countries are shown as colored blocks. *K. p.* subsp. *p., Klebsiella pnueomoniae* subsp. *pneumoniae; K. q.* subsp. *q., Klebsiella quasipneumoniae* subsp. *quasipneumoniae; K. q.* subsp. *s., Klebsiella quasipneumoniae* subsp. *similipneumoniae*.

associated bla_{GES-24} genes from wastewater treatment plant effluent in Japan. The remaining two plasmids in this study, a Col(pWES)/IncP6/IncQ2 plasmid with *intI1*-bla_{GES-24} and a non-typeable plasmid with *intI1*-bla_{GES-5}, were genetically related to the latter IncF plasmids.

3.6. Analysis of bla_{VEB-1}-harboring plasmids

The bla_{VEB-1} gene identified in *K. pneumoniae* subsp. *pneumoniae* mS2H5 and *K. quasipneumoniae* subsp. *similipneumoniae* mS2H7 was located on non-typeable plasmids pmS2H5VEB-1 and pmS2H7-VEB1 with sizes of 21,462 and 22,029 bp, respectively (Table 4). Comparative analysis of the complete sequences of pmS2H5VEB-1 and pmS2H7-VEB1 revealed that they shared 100% nucleotide sequence identity, except for a 567-bp region containing the *dfrA14* gene harbored by pmS2H7-VEB1 (Fig. 3). In these two plasmids, 13.7-kb Tn*402*-like transposon with IS*6100* and no *tni* module, flanked by inverted repeat sequences IRi and IRt with 5-bp direct repeats (AAAAC), were inserted into the plasmid backbone consisting of genes involved in conjugation (*traD* and *mobA*) and replication (*repA* and *repF*). The defective Tn*402*-like transposon carried a new class 1 integron, In1883, containing gene cassettes mediating multiple-drug resistance (*intI1-qnrVC4-qacH4-aacA4-*Δ*cmlA7-tnpA-orf1-orf2-tnpR-*Δ*cmlA7-*bla_{VEB-1}*-aadB*), where the chloramphenicol resistance gene, *cmlA7*, was interrupted by a 4.9-kb Tn*3* family transposon (*tnpA-orf1-orf2-tnpR*).

The plasmid pmS2H7-VEB1 showed 99.9% sequence identity (75% query coverage) to pRHBSTW-00138_5 (CP058135) harbored by *K. quasipneumoniae* subsp. *similipneumoniae* RHBSTW-00138 from wastewater influent in the United Kingdom and 100% sequence identity (54% query coverage) to pWP2-S18-CRE-03_2 (AP021942) harbored by *Aeromonas veronii* WP2-S18-CRE-03 from wastewater effluent in Japan (Fig. 3). *Escherichia coli* NEB10-

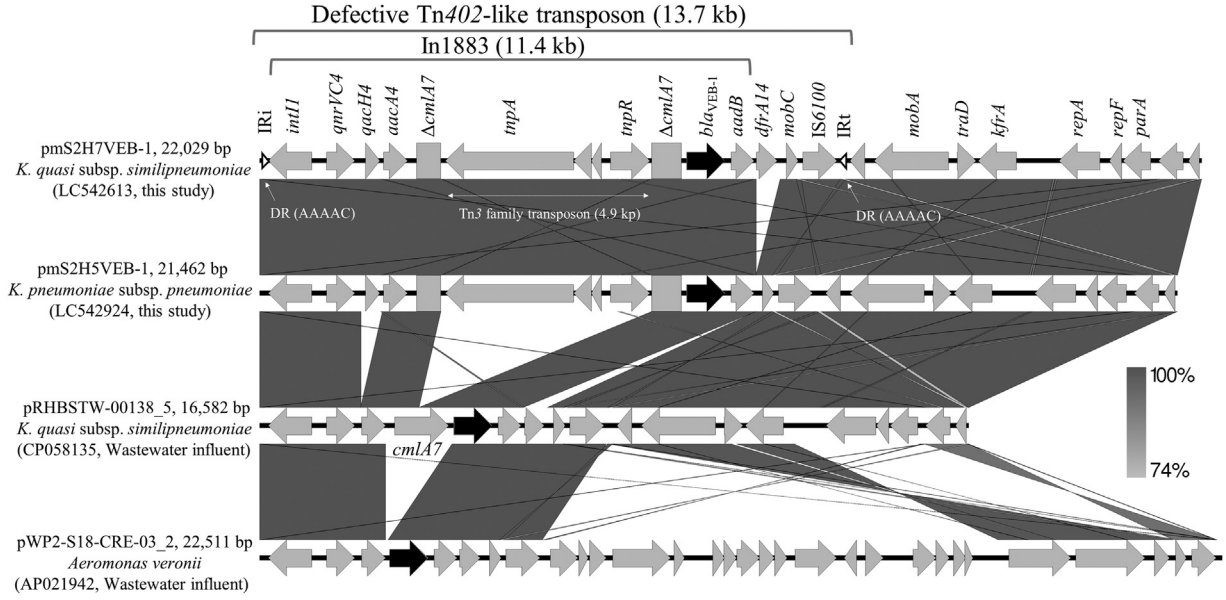

Fig. 3. Linear comparison of complete plasmid sequences of bla_{VEB-1}-carrying plasmid pmS2H7VEB-1 (this study, accession no. LC542613), pmS2H5VEB-1 (this study, LC542924), pRHBSTW-00138_5 (CP058135), and pWP2-S18-CRE-03_2 (AP021942). The bla_{VEB-1} genes are indicated by solid black arrows.

beta transformants that had acquired bla_{VEB-1}-carrying plasmids, pmS2H5VEB-1 and pmS2H7-VEB1, showed higher MICs to ceftazidime (MIC >16 μg/mL), cefpodoxime (MIC >4 μg/mL), and aztreonam (MIC >16 μg/mL) than those for *E. colli* NEB10-beta recipient strain, while the MICs to carbapenems (imipenem, meropenem, and ertapenem) for the transformants remained unchanged (Supplementary Table S2).

4. Discussion

In the present study, we confirmed the existence of bla_{GES-24}- and bla_{GES-5}-carrying *K. pneumoniae* species complex, *E. cloacae* complex, and *P. aeruginosa* isolates in the hospital wastewater effluent samples over the course of three months.

Among the GES-type class A β-lactamases, both GES-24 and GES-5 with amino acid substitution of Gly170Ser within the Ω-loop region display carbapenem-hydrolyzing activities. The relatively lower carbapenem MICs in some isolates of GES carbapenemase producers [18,19] combined with no available selective inhibitors specific for GES carbapenemases may make it challenging to detect these enzymes, leading to an underestimation of the actual prevalence of GES carbapenemase-producing isolates. In this study, all *Enterobacterales* isolates (except *K. pneumoniae* subsp. *pneumoniae* mS2H5 and *K. quasipneumoniae* subsp. *similipneumoniae* mS2H7) co-harboring bla_{GES-24} and bla_{VEB-1} were carbapenemase-negative by mCIM, resulting in false-negative results, while *P. aeruginosa* IPM3H3 carrying bla_{GES-5} was carbapenemase-positive by CIMTris (data not shown) [20]. However, the rapid and accurate identification of GES carbapenemase producers in a timely manner is of clinical significance because they severely limit treatment options [18], cause disease with fatal outcomes despite treatment with polymyxin B and tigecycline [21], and cause the repeated occurrence of outbreaks [19].

Although rare in clinical isolates, GES carbapenemases have been identified worldwide, whereas the GES-5 producers have been reported in *Enterobacterales*, *P. aeruginosa*, and *Acinetobacter baumannii* isolates in various countries [22]. More recently, outbreaks of GES-5 producers have been reported in *P. aeruginosa* and *Serratia marcescens* in Japan [23,24], in *Klebsiella oxytoca* in the United Kingdom [19], and in *K. pneumoniae* in South Korea and Poland [25,26]. In contrast, GES-24 carbapenemase-producing clinical isolates have so far been reported only in *E. kobei* and *Aeromonas hydrophila* from Japan and in *P. aeruginosa* from South Korea [27,28,29]. The presence of Gram-negative bacteria producing GES carbapenemases, with GES-5 being the most prevalent in aquatic environments, including hospital sewage and wastewater treatment plants (WWTPs), has been recognized in many countries [30]. In Japan, besides GES-5, GES-24 has also been reported in a *Klebsiella* isolate from WWTP and an *A. hydrophila* isolate from hospital sewage [31,32]. Surprisingly, in this study, all bacterial isolates with reduced susceptibility to carbapenems detected from hospital wastewater specimens were found to be GES-5 or GES-24 producers from diverse species of bacteria. Among them, bla_{GES-24}-harboring *K. quasipneumoniae* subsp. *quasipneumoniae* IPM1H5 and mS1O1, *K. quasipneumoniae* subsp. *similipneumoniae* mS2H7, and *E. kobei* IPM1H4, and bla_{GES-5}-harboring *E. roggenkampii* IPM1H6, were repeatedly detected over two or three months in the hospital wastewater, suggesting continuous inflow of these organisms of human origin entering the sewage drainage system, or their residence in the drainage system. However, the GES carbapenemase producers have so far not been detected, at least from clinical specimens of patients in the study hospital (data not shown).

The present study revealed that the bla_{GES-24} and bla_{GES-5} genes were situated in different class 1 and class 3 integrons containing various gene cassettes located on plasmids. The class 1 integrons harboring antimicrobial resistance genes have played a significant role in the acquisition and global dissemination of multidrug resistance among Gram-negative bacteria of clinical and environmental origin [33]. The bla_{GES} genes are mainly associated with class 1 integrons in clinically relevant *Enterobacterales* [34]. In Japan, the bla_{GES-24} genes within class 1 integrons have been detected in plasmids harbored by *A. hydrophila* and *P. aeruginosa* clinical isolates [23,28]. Also, the class 1 integron-located bla_{GES-24} and bla_{GES-5} genes have been identified in IncP6 plasmid in *K. varicola* isolates and in Col(pWES) plasmid in *K. pneumoniae* isolates, respectively, recovered from the wastewater influent samples at a municipal wastewater treatment plant [31]. The bla_{GES-24} genes within a class 1 integron have also been reported in *A. hydrophila* recovered from hospital sewage [32]. We obtained somewhat different results in that class 3 integrons are predominantly associ-

Table 4
Genetic features of bla_{GES-24}-, bla_{GES-5}-, and bla_{VEB-1}-positive plasmids

Strain	mS1O1	mS1O2	IPM1H5	IPM1H4	IPM1H6	mS2H5	mS2H7	IPM3H3
Species	*Klebsiella quasipneumoniae* subsp. *quasipneumoniae* ST526	*K. quasi* subsp. *similipneumoniae* ST1770	*K. quasi* subsp. *quasipneumoniae* ST4444	*Enterobacter kobei* ST910	*E. roggenkampii* ST963	*K. pneumoniae* subsp. *pneumoniae* ST12	*K. quasi* subsp. *Similipneumoniae* ST4455	*Pseudomonas aeruginosa* ST3364
bla_{GES} plasmid								
Plasmid name	pmS1O1-GES24	pmS1O2-GES24	pIPM1H5-GES24	pIPM1H4-GES24	pIPM1H6-GES5	pmS2H5-GES24_1 pmS2H5-GES24_2	pmS2H7-GES24	pIPM3H3-GES5
bla_{GES} gene	bla_{GES-24} (2 copies)	bla_{GES-24}	bla_{GES-24} (2 copies)	bla_{GES-24} (2 copies)	bla_{GES-5}	bla_{GES-24} (2 copies) bla_{GES-24} (2 copies)	bla_{GES-24} (3 copies)	bla_{GES-5}
Size	31,970 bp	incomplete	60,311 bp	18,031 bp	27,002 bp	129,459 bp 20,476 bp	59,060 bp	36,999 bp
Inc types	Col(pHAD28)	IncFII(pBK30683)	Col(pHAD28)/IncFII(pBK30683)	Col(pHAD28)	non-typeable	Col(pHAD28)/IncFII(pBK30683) Col(pHAD28)	Col(pWES)/IncP6/IncQ2	non-typeable
Conjugation frequency[a] (CFU/donor cell)	4.9×10^{-7}	3.0×10^{-6}	4.1×10^{-6}	–[b]	–[b]	–[b] 8.3×10^{-6}	2.1×10^{-6}	–[b]
bla_{VEB} plasmid								
Plasmid name						pmS2H5VEB-1	pmS2H7VEB-1	
bla_{VEB} gene						bla_{VEB-1}	bla_{VEB-1}	
Size						21,462 bp	22,029 bp	
Inc types						non-typeable	non-typeable	

[a] Conjugation efficiencies were determined by dividing the number of transconjugants by that of the donors.
[b] No transconjugants were recovered.

ated with the bla_{GES-24} gene. Furthermore, a total of five new class 3 integrons, In3-18 to In3-22, were identified among five bla_{GES-24} and one bla_{GES-5} plasmids. So far, 17 class 3 integron numbers, In3-1 to In3-17, have been assigned to the INTEGRALL database (accessed June 2022), among which In3-1 and In3-12 have been reported, respectively, in *Serratia marcescens* and *Pseudomonas fulva* clinical isolates harboring bla_{IMP-1} genes in Japan [35,36]. New class 3 integrons identified in this study mostly carried two tandem copies of bla_{GES-24}, which were harbored by *K. quasipneumoniae* subsp. q*uasipneumoniae, K. pneumoniae* subsp. *pneumoniae*, and *E. kobei*. No correlation was observed between copy numbers of the bla_{GES-24} gene and the MICs of most β-lactams, including imipenem, among these isolates.

The bla_{VEB-1} gene has been reported among members of the *Enterobacterales*, non-fermenting Gram-negative bacteria (particularly *Pseudomonas* spp. in Asia, Europe, and America) [37]. In Japan, there has been only one report of bla_{VEB-3}: it was detected in *A. hydrophila* isolates from river water running through Gifu City, of the same prefecture where the hospital in this study is located [38]. Thus, this study identified a very rare bla_{VEB-1} gene, which was carried by the defective Tn*402*-like class 1 integron located on a non-typeable plasmid in both *K. pneumoniae* subsp. *pneumoniae* mS2H5 and *K. quasipneumoniae* subsp. *similipneumoniae* mS2H7 strains harboring bla_{GES-24} plasmids. The complete sequence of the bla_{VEB-1} plasmid harbored by *K. quasipneumoniae* subsp. *similipneumoniae* mS2H7 showed 100% nucleotide sequence identity to that harbored by *K. pneumoniae* subsp. *pneumoniae* mS2H5, except for the *dfrA14* gene cassette region, suggesting that the acquisition of bla_{VEB-1} genes among these strains harboring bla_{GES-24} plasmids is possibly a result of horizontal plasmid transfer in the hospital wastewater. New class 1 integron, In1883, carrying the bla_{VEB-1} gene, contained many antibiotic resistance gene cassettes, including the quinolone resistance gene *qnrVC4*. The *qnrVC* alleles have often been associated with bacteria from aquatic environments, such as *Aeromonas* and *Vibrio* species; however, the presence of these genes in other bacterial species, including *P. aeruginosa, A. baumannii, Citrobacter freundii*, and *Salmonella enterica* serovar Rissen, has also been recognized [39–41]. In Japan, sequence data are only available for the *qnrVC4* gene in *A. veronii* (AP021942) and *Vibrio cholerae* (AP014525). Interestingly, the resistance gene cassette configuration of a new class 1 integron, In1883 (*qnrVC4-qacH4-aacA4-*Δ*cmlA7-tnpA-orf1-orf2-tnpR-*Δ*cmlA7-*bla_{VEB-1}*-aadB*), followed by *dfrA14-mobC*-IS*6100*, identified in *K. quasipneumoniae* subsp. *similipneumoniae* mS2H7, was similar to that of a class 1 integron, In805, carrying *qnrVC4* (*qnrVC4-qacH4-aacA4-cmlA7-*bla_{OXA-10}*-aadA1-dfrA14-mobC*-IS*6100*) in *S. enterica* serovar Rissen from Thailand [41].

In conclusion, our findings depict the predominance of bla_{GES-24} among CPO isolates in hospital wastewater habitats. The bla_{GES-24} genes were mostly embedded in new complete or defective Tn*402*-like class 3 integrons located on plasmids of Col(pHAD28), IncFII(pBK30683), and Col(pHAD28)/IncFII(pBK30683), which were carried by *Klebsiella* spp. and *Enterobacter* spp. The importance of Tn*402*-like class 1 integron in the accumulation of resistance genes and their spreading in human pathogens has been recognized [42]. This study confirmed the linkage of bla_{GES-24} genes with rare Tn*402*-like class 3 integrons and the structural diversity of their gene cassette arrays, suggesting a plasticity inherent to these integrons that enables their capture of additional resistance gene cassettes in hospital wastewater environments. A major concern raised by the presence and persistence of these GES-5- and GES-24-positive pathogenic Gram-negative bacteria in hospital wastewater is the potential spread of resistant isolates or resistance genes/plasmids into the hospital environment from contaminated pipework and sinks, leading to increased risk of nosocomial transmission.

Competing interests

None declared

Funding

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Ethical approval

Not required

Sequence information

The complete genome sequences of eight strains have been deposited to NCBI BioProject under accession number PRJNA666944. The nucleotide sequences of plasmids have been deposited in the NCBI under the accession numbers LC542613, LC542924, LC589061-589064, LC620536, LC621169, LC622104, LC623933, and LC634084.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jgar.2022.09.005.

References

[1] Naas T, Dortet L, Iorga BI. Structural and functional aspects of class A carbapenemases. Curr Drug Targets 2016;17:1006–28. doi:10.2174/1389450117666160310144501.
[2] Diene SM, Rolain JM. Carbapenemase genes and genetic platforms in Gram-negative bacilli: *Enterobacteriaceae, Pseudomonas* and *Acinetobacter* species. Clin Microbiol Infect 2014;20:831–8. doi:10.1111/1469-0691.12655.
[3] Woodford N, Turton JF, Livermore DM. Multiresistant Gram-negative bacteria: the role of high-risk clones in the dissemination of antibiotic resistance. FEMS Microbiol Rev 2011;35:736–55. doi:10.1111/j.1574-6976.2011.00268.x.
[4] Cahill N, O'Connor L, Mahon B, Varley Á, McGrath E, Ryan P, et al. Hospital effluent: a reservoir for carbapenemase-producing *Enterobacterales*? Sci Total Environ 2019;672:618–24. doi:10.1016/j.scitotenv.2019.03.428.
[5] Hassoun-Kheir N, Stabholz Y, Kreft JU, de la Cruz R, Romalde JL, Nesme J, et al. Comparison of antibiotic-resistant bacteria and antibiotic resistance genes abundance in hospital and community wastewater: a systematic review. Sci Total Environ 2020;743:140804. doi:10.1016/j.scitotenv.2020.140804.
[6] Khan FA, Hellmark B, Ehricht R, Söderquist B, Jass J. Related carbapenemase-producing *Klebsiella* isolates detected in both a hospital and associated aquatic environment in Sweden. Eur J Clin Microbiol Infect Dis 2018;37:2241–51. doi:10.1007/s10096-018-3365-9.
[7] Kizny Gordon AE, Mathers AJ, Cheong EYL, Gottlieb T, Kotay S, Walker AS, et al. The hospital water environment as a reservoir for carbapenem-resistant organisms causing hospital-acquired infections-a systematic review of the literature. Clin Infect Dis 2017;64:1435–44. doi:10.1093/cid/cix132.
[8] Hopman J, Meijer C, Kenters N, Coolen JPM, Ghamati MR, Mehtar S, et al. Risk assessment after a severe hospital-acquired infection associated with carbapenemase-producing *Pseudomonas aeruginosa*. JAMA Netw Open 2019;2:e187665. doi:10.1001/jamanetworkopen.2018.7665.
[9] Hoffmann H, Roggenkamp A. Population genetics of the nomenspecies *Enterobacter cloacae*. Appl Environ Microbiol 2003;69:5306–18. doi:10.1128/AEM.69.9.5306-5318.2003.
[10] Paauw A, Caspers MP, Schuren FH, Leverstein-van Hall MA, Delétoile A, Montijn RC, et al. Genomic diversity within the *Enterobacter cloacae* complex. PLoS One 2008;3:e3018. doi:10.1371/journal.pone.0003018.
[11] Hayashi W, Izumi K, Yoshida S, Takizawa S, Sakaguchi K, Iyori K, et al. Antimicrobial resistance and type III secretion system virulotypes of *Pseudomonas aeruginosa* isolates from dogs and cats in primary veterinary hospitals in Japan: identification of the international high-risk clone sequence type 235. Microbiol Spectr 2021;9:e0040821. doi:10.1128/Spectrum.00408-21.
[12] Hayashi W, Yoshida S, Izumi K, Koide S, Soga E, Takizawa S, et al. Genomic characterisation and epidemiology of nosocomial *Serratia marcescens* isolates resistant to ceftazidime and their plasmids mediating rare bla_{TEM-61}. J Glob Antimicrob Resist 2021;25:124–31. doi:10.1016/j.jgar.2021.03.010.
[13] Clinical and Laboratory Standards Institute Performance standards for antimicrobial susceptibility testing. CLSI document M100-Ed30. Wayne, PA: Clinical and Laboratory Standards Institute; 2020.
[14] Wick RR, Judd LM, Gorrie CL, Holt KE. Unicycler: resolving bacterial genome assemblies from short and long sequencing reads. PLoS Comput Biol 2017;13:e1005595. doi:10.1371/journal.pcbi.1005595.
[15] Lees JA, Harris SR, Tonkin-Hill G, Gladstone RA, Lo SW, Weiser JN, et al. Fast and flexible bacterial genomic epidemiology with PopPUNK. Genome Res 2019;29:304–16. doi:10.1101/gr.241455.118.
[16] Madsen JS, Riber L, Kot W, Basfeld A, Burmølle M, Hansen LH, et al. Type 3 fimbriae encoded on plasmids are expressed from a unique promoter without affecting host motility, facilitating an exceptional phenotype that enhances conjugal plasmid transfer. PLoS One 2016;11:e0162390. doi:10.1371/journal.pone.0162390.
[17] Hayashi W, Iimura M, Soga E, Koide S, Izumi K, Yoshida S, et al. Presence of colistin- and tigecycline-resistant *Klebsiella pneumoniae* ST29 in municipal wastewater influents in Japan. Microb Drug Resist 2021;27:1433–42. doi:10.1089/mdr.2020.0514.
[18] Bonnin RA, Jousset AB, Urvoy N, Gauthier L, Tlili L, Creton E, et al. Detection of GES-5 carbapenemase in *Klebsiella pneumoniae*, a newcomer in France. Antimicrob Agents Chemother 2017;61:e02263 –16. doi:10.1128/AAC.02263-16.
[19] Ellington MJ, Davies F, Jauneikaite E, Hopkins KL, Turton JF, Adams G, et al. A multispecies cluster of GES-5 carbapenemase-producing *Enterobacterales* linked by a geographically disseminated plasmid. Clin Infect Dis 2020;71:2553–60. doi:10.1093/cid/ciz1130.
[20] Uechi K, Tada T, Shimada K, Kuwahara-Arai K, Arakaki M, Tome T, et al. A modified carbapenem inactivation method, CIMTris, for carbapenemase production in *Acinetobacter* and *Pseudomonas* species. J Clin Microbiol 2017;55:3405–10. doi:10.1128/JCM.00893-17.
[21] Ribeiro VB, Falci DR, Rozales FP, Barth AL, Zavascki AP. Carbapenem-resistant GES-5-producing *Klebsiella pneumoniae* in Southern Brazil. Braz J Infect Dis 2014;18:231–2. doi:10.1016/j.bjid.2013.12.002.
[22] Nordmann P, Poirel L. The difficult-to-control spread of carbapenemase producers among *Enterobacteriaceae* worldwide. Clin Microbiol Infect 2014;20:821–30. doi:10.1111/1469-0691.12719.
[23] Hishinuma T, Tada T, Kuwahara-Arai K, Yamamoto N, Shimojima M, Kirikae T. Spread of GES-5 carbapenemase-producing *Pseudomonas aeruginosa* clinical isolates in Japan due to clonal expansion of ST235. PLoS One 2018;13:e0207134. doi:10.1371/journal.pone.0207134.
[24] Nakanishi N, Komatsu S, Iwamoto T, Nomoto R. Characterization of a novel plasmid in *Serratia marcescens* harbouring bla_{GES-5} isolated from a nosocomial outbreak in Japan. J Hosp Infect 2022;121:128–31. doi:10.1016/j.jhin.2021.11.022.
[25] Jeong SH, Bae IK, Kim D, Hong SG, Song JS, Lee JH, et al. First outbreak of *Klebsiella pneumoniae* clinical isolates producing GES-5 and SHV-12 extended-spectrum beta-lactamases in Korea. Antimicrob Agents Chemother 2005;49:4809–10. doi:10.1128/AAC.49.11.4809-4810.2005.
[26] Literacka E, Izdebski R, Urbanowicz P, Żabicka D, Klepacka J, Sowa-Sierant I, et al. Spread of *Klebsiella pneumoniae* ST45 producing GES-5 carbapenemase or GES-1 extended-spectrum β-lactamase in newborns and infants. Antimicrob Agents Chemother 2020;64:e00595 –20. doi:10.1128/AAC.00595-20.
[27] Nakamura A, Nakamura T, Niki M, Kuchibiro T, Nishi I, Komatsu M. Genomic characterization of ESBL- and carbapenemase-positive *Enterobacteriaceae* co-harboring *mcr-9* in Japan. Front Microbiol 2021;12:665432. doi:10.3389/fmicb.2021.665432.
[28] Uechi K, Tada T, Sawachi Y, Hishinuma T, Takaesu R, Nakama M, et al. A carbapenem-resistant clinical isolate of *Aeromonas hydrophila* in Japan harbouring an acquired gene encoding GES-24 β-lactamase. J Med Microbiol 2018;67:1535–7. doi:10.1099/jmm.0.000842.
[29] Hong JS, Choi N, Kim SJ, Choi KH, Roh KH, Lee S. Molecular characteristics of GES-type carbapenemase-producing *Pseudomonas aeruginosa* clinical isolates from long-term care facilities and general hospitals in South Korea. Microb Drug Resist 2020;26:605–10. doi:10.1089/mdr.2019.0302.
[30] Cherak Z, Loucif L, Moussi A, Rolain JM. Carbapenemase-producing Gram-negative bacteria in aquatic environments: a review. J Glob Antimicrob Resist 2021;25:287–309. doi:10.1016/j.jgar.2021.03.024.
[31] Gomi R, Matsuda T, Yamamoto M, Chou PH, Tanaka M, Ichiyama S, et al. Characteristics of carbapenemase-producing *Enterobacteriaceae* in wastewater revealed by genomic analysis. Antimicrob Agents Chemother 2018;2:e02501–17. doi:10.1128/AAC.02501-17.
[32] Maehana S, Eda R, Hirabayashi A, Niida N, Nakamura M, Furukawa T, et al. Natural factories that manufacture antimicrobial resistance genes: quadruple bla_{GES}-carrying plasmids in *Aeromonas* and *Pseudomonas* species. Int J Antimicrob Agents 2021;57:106327. doi:10.1016/j.ijantimicag.2021.106327.
[33] Deng Y, Bao X, Ji L, Chen L, Liu J, Miao J, et al. Resistance integrons: class 1, 2 and 3 integrons. Ann Clin Microbiol Antimicrob 2015;14:45. doi:10.1186/s12941-015-0100-6.
[34] Pedersen T, Sekyere JO, Govinden U, Moodley K, Sivertsen A, Samuelsen Ø, et al. Spread of plasmid-encoded NDM-1 and GES-5 carbapenemases among extensively drug-resistant and pandrug-resistant clinical *Enterobacteriaceae* in Durban, South Africa. Antimicrob Agents Chemother 2018;62:e02178 –17. doi:10.1128/AAC.02178-17.
[35] Collis CM, Kim MJ, Partridge SR, Stokes HW, Hall RM. Characterization of the class 3 integron and the site-specific recombination system it determines. J Bacteriol 2002;184:3017–26. doi:10.1128/JB.184.11.3017-3026.2002.
[36] Yamamoto M, Matsumura Y, Gomi R, Matsuda T, Nakano S, Nagao M, et al. Molecular analysis of a bla_{IMP-1}-harboring class 3 integron in multidrug-resistant *Pseudomonas fulva*. Antimicrob Agents Chemother 2018;62:e00701–18. doi:10.1128/AAC.00701-18.
[37] Naas T, Poirel L, Nordmann P. Minor extended-spectrum beta-lactamases. Clin Microbiol Infect 2008(Suppl 1):42–52. doi:10.1111/j.1469-0691.2007.01861.x.

[38] Aratani T, Koide N, Hayami K, Sugiyama M, Minamoto T, Asai T. Continuous prevalence of VEB-3 extended-spectrum β-lactamase-producing *Aeromonas hydrophila* in a local river in Gifu City. Japan. Microbiol Immunol 2021;5:99–100. doi:10.1111/1348-0421.12873.
[39] Ye L, Li R, Lin D, Zhou Y, Fu A, Ding Q, et al. Characterization of an IncA/C multidrug resistance plasmid in *Vibrio alginolyticus*. Antimicrob Agents Chemother 2016;60:3232–5. doi:10.1128/AAC.00300-16.
[40] Zhu Y, Liu L, Schwarz S, Liu W, Wang C, Yang Q, et al. Characterization of a novel hybrid plasmid coharboring bla_{KPC-2} and *qnrVC4* in a clinical *Citrobacter freundii* strain. Antimicrob Agents Chemother 2020;64:e01379 –20. doi:10.1128/AAC.01379-20.
[41] Luk-In S, Pulsrikarn C, Bangtrakulnonth A, Chatsuwan T, Kulwichit W. Occurrence of a novel class 1 integron harboring *qnrVC4* in *Salmonella* Rissen. Diagn Microbiol Infect Dis 2017;88:282–6. doi:10.1016/j.diagmicrobio.2017.03.016.
[42] Gillings MR, Labbate M, Sajjad A, Giguère NJ, Holley MP, Stokes HW. Mobilization of a Tn*402*-like class 1 integron with a novel cassette array via flanking miniature inverted-repeat transposable element-like structures. Appl Environ Microbiol 2009;75:6002–4. doi:10.1128/AEM.01033-09.