Title

Characterization of a Novel Plasmid in *Serratia marcescens* Harboring bla_{GES-5} Isolated from a Nosocomial Outbreak in Japan

Running title

Plasmid in *S. marcescens* Confers Carbapenem Resistance

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Abstract

Serratia marcescens is a nosocomial pathogen with carbapenem resistance, limiting the availability of effective treatment options. In this study, we performed molecular characterization of GES-5 carbapenemase-producing *S. marcescens* isolated from an outbreak in Japan. Comparative genetic analysis revealed that the *bla*$_{GES-5}$–encoding plasmid p2020-O-9 is a unique plasmid contributing towards carbapenem resistance. Furthermore, this study highlights the necessity of surveillance programs for monitoring novel, along with commonly occurring carbapenemases in clinical settings.

KEYWORDS: *Serratia marcescens*, crabapenemase, *bla*$_{GES-5}$, whole-genome sequencing

Abbreviations: WGS: whole-genome sequencing

Serratia marcescens is a major opportunistic pathogen known to cause nosocomial infections associated with high morbidity and mortality (1, 2). As this bacterium shows resistance towards widely used carbapenem class of antibiotics, treatment options available for nosocomial infections becomes limited (3-5). Carbapenemases (a class of β-lactamases) identified in Japan comprise KPC, GES, NDM, IMP, VIM, and OXA-48 variants, with IMP being the most prevalent in the country (6). GES type Amber class A β-lactamases, in addition to exhibiting β-lactam resistance, also confer carbapenemase activity, such as the GES-5 variant (7, 8). In recent times, nosocomial outbreaks have largely been attributed to GES-5 carbapenemase-producing bacterial strains in various countries including Japan, wherein the first outbreak of *P. aeruginosa* harboring the $bla_{\text{GES-5}}$ gene occurred in the year 2014 (9-13). This study reports the genetic characteristics of a different GES-5 producing

bacterium, *S. marcescens* isolated from an ICU outbreak in Japan in 2020.

A total of six carbapenem-resistant *S. marcescens* strains were isolated from the samples from three ICU patients collected during May-October 2020 (Table 1). Strain was identified using MALDI Biotyper (Bruker Daltonics K.K., Yokohama, Japan), and the minimal inhibitory concentrations (MICs) of various antimicrobial agents were determined by E-test (bioMérieux Japan Ltd., Tokyo, Japan) following the manufacturer's guidelines. As indicated in Table 1, all of the six *S. marcescens* strains were resistant to imipenem, meropenem, and ceftazidime. Modified carbapenem inactivation method (mCIM) indicated the absence of carbapenemase genes in all the strains. This was supported by the negative results of PCR screening conducted for the major carbapenemase genes bla_{IMP}, bla_{NDM}, bla_{KPC}, and $bla_{\mathrm{OXA\text{-}48\text{-}like}}$; however, a positive result was obtained for bla_{GES}. The MICs of antimicrobial agents (except ampicillin (AM) and cefotaxime (CTX)) in 2020-O-14-2 strain lacking the $bla_{\mathrm{GES\text{-}5}}$ gene, isolated from patient B, were significantly lower than all the other

53 isolates. Whole-genome sequencing of the six isolates harboring *bla*$_{\text{GES-5}}$ gene and the one
54 lacking the gene was done for analyzing single nucleotide variations and deriving genetic
55 relationships among the seven isolates to understand the mechanisms of antimicrobial
56 resistance. A survey for analyzing environmental contamination in the ICU was conducted
57 during the outbreak period; however, the presence of carbapenem-resistant *S.*
58 *marcescens* strains was not detected.

59 Genomic libraries of all seven isolates were prepared using the Nextera XT DNA library
60 prep kit (Illumina, San Diego, CA, USA) and sequenced on the iSeq 100 system (Illumina).
61 Additionally, Oxford Nanopore Technologies (ONT) were used for constructing a
62 sequencing library of the strain 2020-O-9 with the help of Rapid Barcoding Kit
63 (SQK-RBK004), and sequenced on a MinION device using flow cell type R9.4.1
64 (FLO-MIN106D). Hybrid assembly of 2020-O-9 genome was performed using Unicycler

v0.4.8 (14), and a circular chromosome and plasmid (p2020-O-9) sequence was obtained. The genome sequences were annotated using DFAST (https://dfast.nig.ac.jp/) and are available in DDBJ (DNA Data Bank of Japan) under the accession numbers AP024847 (chromosome) and AP024848 (plasmid). SNP analysis of the remaining 6 strains was performed taking the genome sequence of 2020-O-9 as a reference, using bwa version 0.7.17, samtools version 1.9, and VarScan v2.4.4 (15-17). The analysis revealed high degree of genetic homogeneity with 0 to 5 SNPs consistent among the seven strains isolated, including GES-5 negative strain 2020-O-14-2. The whole-genome sequencing reads are available in DDBJ Sequence Read Archive under the accession number listed in Table 1. Since MICs of carbapenems in 2020-O-14-2 strain lacking the *bla*$_{\text{GES-5}}$ gene were significantly decreased, it is evident that the newly detected *bla*$_{\text{GES-5}}$ containing plasmid contributes to carbapenem

resistance.

Further genetic characterization of p2020-O-9 was done using ResFinder 3.2 to identify resistance genes, and plasmid incompatibility replicon typing was performed using PlasmidFinder 2.0 developed by the Center for Genomic Epidemiology (http://www.genomicepidemiology.org/). The chromosomal sequence of 2020-O-9 consisted of a single antimicrobial resistance gene $bla_{\text{SRT-2}}$, as identified by existing databases. p2020-O-9 is a novel 23,921-bp circular untypeable plasmid, with the $bla_{\text{GES-5}}$ gene located between intI1 of class 1 integron and the *qacEΔ1* and *sul1*, together with a gene of GNAT-family N-acetyltransferase, *aac(6)-29a*. It has a GC-content of 61% and carries 26 protein-coding genes (Fig.1). Sequence analysis using NCBI revealed that the plasmid backbone of p2020-O-9 showed highest similarity with plasmid pCAV1374-16 from

Klebsiella oxytoca with a query coverage of 59%; however, there was no $bla_{\text{GES-5}}$ in pCAV1374-16 (Accession no. CP011628). The class 1 integron cassette around the $bla_{\text{GES-5}}$ gene in p2020-O-9 is similar to that of pMRY16-414SMA_2 from *S. marcescens* (Accession no. LC486677) and the plasmid from *Aeromonas hydrophila* strain WCHAH 01-derived plasmids pGES5 (Accession no. KR014105) (Fig. 1). Plasmid sequences similar to the backbone of p2020-O-9 such as pCAV1374-16, have been registered in databases from a wide range of bacterial species. It is believed that $bla_{\text{GES-5}}$ was integrated into the class 1 integron cassette of a plasmid like pCAV1374-16 to construct the novel p2020-O-9 plasmid. This suggests that p2020-O-9 could be an untypeable plasmid with the potential to distribute itself widely in *Enterobacteriaceae*.

In summary, we reported the molecular characterization of GES-5 producing *S. marcescens* strain harboring a novel bla_{GES-5} gene-encoding plasmid, p2020-O-9 isolated from the ICU outbreak in Japan The prevalence and transmission of GES-producing bacteria is largely underestimated due to their rare presence in clinical isolates. However, through this study, we highlight the necessity of surveillance programs for monitoring novel, as well as commonly occurring carbapenemases in clinical settings.

Data availability

Sequence data that support the findings of this study have been deposited in DDBJ (https://www.ddbj.nig.ac.jp/) with the accession numbers AP024847 and AP024848.

Author contributions

NN, RN designed the study methods and wrote the first draft of the manuscript. NN, RN, SK collected the data. NN, RN analyzed the data. TI contributed to the writing of the manuscript.

Ethical approval

This study was approved by the Ethical Review Committee of the Kobe Institute of Health (approval No. SenR3-9).

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We declare no conflicts of interest.

References

1. **Iosifidis E, Farmaki E, Nedelkopoulou N, Tsivitanidou M, Kaperoni M, Pentsoglou V, Pournaras S, Athanasiou-Metaxa M, Roilides E**. 2012. Outbreak of bloodstream infections because of Serratia marcescens in a pediatric department. Am J Infect Control

40:11–15. doi: 10.1016/j.ajic.2011.03.020.

2. **Gastmeier P**. 2014. *Serratia marcescens*: an outbreak experience. Front Microbiol. 2014 **5**:81. doi: 10.3389/fmicb.2014.00081.

3. **Moradigaravand D, Boinett CJ, Martin V, Peacock SJ, Parkhill J**. 2016. Recent independent emergence of multiple multidrug-resistant *Serratia marcescens* clones within the United Kingdom and Ireland. Genome Res. **26**:1101–1109.

4. **Silva KE, Cayô R, Carvalhaes CG, Patussi Correia Sacchi F, Rodrigues-Costa F, Ramos da Silva AC, Croda J, Gales AC, Simionatto S**. 2015. Coproduction of KPC-2 and IMP-10 in carbapenem-resistant *Serratia marcescens* isolates from an outbreak in a Brazilian teaching hospital. J Clin Microbiol **53**:2324–2328. doi: 10.1128/JCM.00727-15.

5. **Nastro M, Monge R, Zintgraff J, Vaulet LG, Boutureira M, Famiglietti A, Rodriguez CH**. 2013. First nosocomial outbreak of VIM-16-producing Serratia marcescens in Argentina. Clin Microbiol Infect.: the Official Publication of the European Society of Clinical Microbiology and Infectious Diseases **19**:617–619. doi: 10.1111/j.1469-0691.2012.03978.x.

6. **Logan LK, Weinstein RA**. 2017. The epidemiology of carbapenem-resistant Enterobacteriaceae: the impact and evolution of a global menace. J Infect Dis. **215**:S28–S36. doi: 10.1093/infdis/jiw282.

7. **Bontron S, Poirel L, Nordmann P**. 2015. In vitro prediction of the evolution of GES-1 β-lactamase hydrolytic activity. Antimicrob. Agents Chemother. **59**:1664–1670.

8. **Walther-Rasmussen J, Høiby N**. 2007. Class A carbapenemases. J Antimicrob

Chemother **60**:470–482.

9. **Ellington MJ, Davies F, Jauneikaite E, Hopkins KL, Turton JF, Adams G, Pavlu J, Innes AJ, Eades C, Brannigan ET, Findlay J, White L, Bolt F, Kadhani T, Chow Y, Patel B, Mookerjee S, Otter JA, Sriskandan S, Woodford N, Holmes A**. 2020. A multispecies cluster of GES-5 carbapenemase-producing Enterobacterales linked by a geographically disseminated plasmid. Clin Infect Dis.: an Official Publication of the Infectious Diseases Society of America **71**:2553–2560. doi: 10.1093/cid/ciz1130.

10. **Chudejova K, Rotova V, Skalova A, Medvecky M, Adamkova V, Papagiannitsis CC, Hrabak J**. 2018. Emergence of sequence type 252 Enterobacter cloacae producing GES-5 carbapenemase in a Czech hospital. Diagn Microbiol Infect Dis **90**:148–150.

11. **Hishinuma T, Tada T, Kuwahara-Arai K, Yamamoto N, Shimojima M, Kirikae T**.

2018. Spread of GES-5 carbapenemase-producing Pseudomonas aeruginosa clinical isolates in Japan due to clonal expansion of ST235. PLoS One **13**:e0207134.

12. **Literacka E, Izdebski R, Urbanowicz P, Żabicka D, Klepacka J, Sowa-Sierant I, Żak I, Garus-Jakubowska A, Hryniewicz W, Gniadkowski M**. 2020. Spread of Klebsiella pneumoniae ST45 Producing GES-5 carbapenemase or GES-1 Extended-Spectrum β-lactamase in Newborns and Infants. Antimicrob Agents Chemother 64:64(9):e00595-20. doi: 10.1128/AAC.00595-20.

13. **Kanayama A, Kawahara R, Yamagishi T, Goto K, Kobaru Y, Takano M, Morisada K, Ukimura A, Kawanishi F, Tabuchi A, Matsui T, Oishi K**. 2016. Successful control of an outbreak of GES-5 extended-spectrum β-lactamase-producing Pseudomonas aeruginosa in a long-term care facility in Japan. J Hosp Infect. **93**:35–41.

14. **Wick RR, Judd LM, Gorrie CL, Holt KE**. 2017. Unicycler: resolving bacterial genome assemblies from short and long sequencing reads. PLoS Comput Biol **13**:e1005595.

15. **Li H**. Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. arXiv:1303.3997v2 [q-bio.GN]. 2013.

16. **Danecek P, Bonfield JK, Liddle J, Marshall J, Ohan V, Pollard MO, Whitwham A, Keane T, McCarthy SA, Davies RM, Li H**. 2021. Twelve years of SAMtools and BCFtools. GigaScience **10**. https://doi.org/10.1093/gigascience/giab008.

17. **Koboldt DC, Zhang Q, Larson DE, Shen D, McLellan MD, Lin L, Miller CA, Mardis ER, Ding L, Wilson RK**. 2012. VarScan 2: Somatic mutation and copy number alteration discovery in cancer by exome sequencing. Genome Research

22:568–576. DOI: 10.1101/gr.129684.111.

18. **Alikhan NF, Petty NK, Ben Zakour NL, Beatson SA**. 2011. BLAST Ring Image Generator (BRIG): simple prokaryote genome comparisons. BMC Genomics **12**:402. PMID: 21824423.

188 **Figure legends**

189 **Figure 1.**

190 Circular representation of p2020-O-9 was generated using BLAST Ring Image Generator

191 0.95 (18). A comparison of the plasmid with pMRY16-414SMA_2 from Serratia marcescens

192 MRY-414SMA (LC486677), pGES5 from Aeromonas hydrophila WCHAH 01

193 (KR014105), and pCAV1374-16 from Klebsiella oxytoca (CP011628). The outermost circle

194 indicates the coding sequence of p2020-O-9. Red, carbapenemase genes; blue, other

195 antimicrobial resistance genes; purple, IS6100; orange, transposase, and recombinase genes;

196 green, integrase genes; grey, other genes or coding sequences.

197

TABLE 1. Information and the antibiotic susceptibility of the *Serratia marcescens* isolated from three patients involved in the ICU outbreaks

Patients	Age (yr), sex[a]	No. of isolate	Date of isolation (year/month/day)	Specimens	MICs (μg/ml) for[b]													*bla*$_{GES-5}$	DDBJ accession no. of read data
					AM	CTX	CAZ	FEP	ATM	IMP	MPM	DOR	ETP	AK	MC	CIP	PMB		
A	76, F	2020-O-9	2020/5/25	Sputum	>256	>32	>32	6	6	>32	>32	>32	>32	>256	4	0.5	>1024	+	DRR308222
		2020-O-12	2020/6/23	Blood	>256	>32	>32	3	4	>32	>32	>32	>32	>256	3	1	>1024	+	DRR308223
		2020-O-21-2	2020/8/3	Sputum	>256	>32	>32	2	3	6	>32	>32	8	64	3	1.5	64	+	DRR308224
		2020-O-21-3	2020/8/4	Blood	>256	>32	>32	2	8	>32	>32	24	>32	96	3	1.5	128	+	DRR308225
B	70, M	2020-O-14-1	2020/6/19	Blood	>256	>32	>32	4	6	>32	>32	>32	>32	128	12	>32	96	+	DRR308226
		2020-O-14-2			>256	>32	2	3	4	1	1.5	1.5	4	16	3	3	64	-	DRR308227
C	59, M	2020-O-25	2020/10/15	Ascites	>256	>32	>32	3	8	>32	>32	>32	>32	>256	3	0.38	>1024	+	DRR308228

[a] F, feamale, M, male.

[b]ampicillin (AM), cefotaxime (CTX), ceftazidime (CAZ), cefepime (FEP), aztreonam (ATM), imipenem (IMP), meropenem (MPM), doripenem (DOR), ertapenem (ETP), amikacin (AK), minocycline (MC), ciprofloxacin (CIP), polymyxin B (PMB)

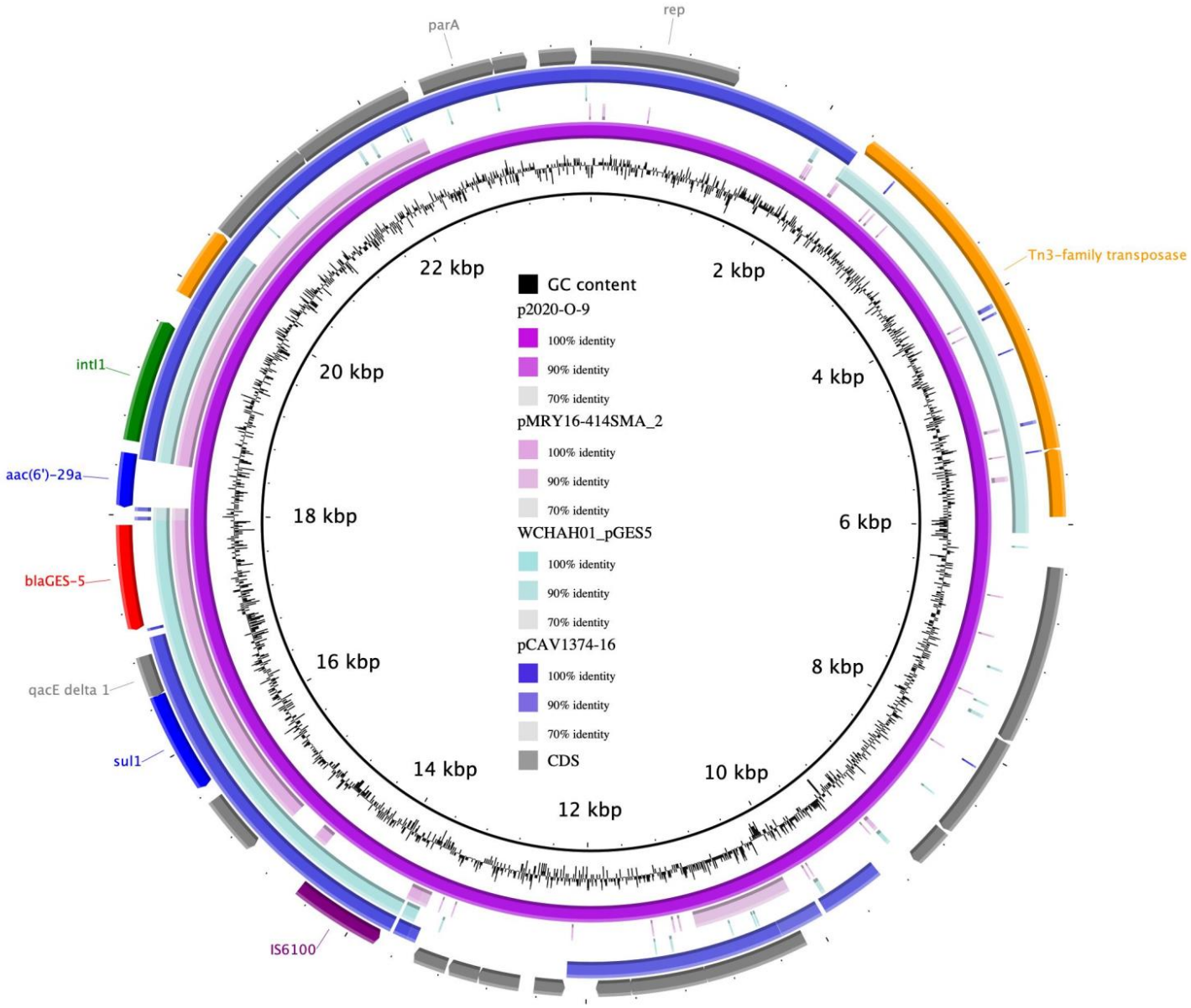

Figure 1. Circular representation of p2020-O-9 was generated using BLAST Ring Image Generator 0.95 (18). A comparison with the pMRY16-414SMA_2 from *Serratia marcescens* MRY-414SMA (LC486677), pGES5 from *Aeromonas hydrophila* WCHAH 01 (KR014105), and pCAV1374-16 from *Klebsiella oxytoca* (CP011628). The outermost circle shows the coding sequence of p2020-O-9. Red, carbapenemase genes; blue, other antimicrobial resistance genes; purple, IS6100; orange, transposase and recombinase genes; green, integrase genes; grey, other genes or coding sequences.