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Genomic characterisation and epidemiology of nosocomial *Serratia marcescens* isolates resistant to ceftazidime and their plasmids mediating rare $bla_{\text{TEM-61}}$

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ABSTRACT

Objectives: We determined the whole DNA sequences of plasmids carrying a rare extended-spectrum β-lactamase gene ($bla_{\text{TEM-61}}$) to precisely understand the spread of resistance among nosocomial *Serratia marcescens* populations.

Methods: Twenty non-duplicate ceftazidime-resistant *S. marcescens* nosocomial isolates (ceftazidime MICs, 32 to >128 mg/L) collected over 1 year were pulsotyped and nucleotide sequences of the $bla_{\text{TEM-61}}$ gene and its promoter region were determined. Twelve representative isolates were analysed by whole-genome sequencing.

Results: The 20 isolates comprised two distinct pulsotypes: I (14 isolates) and II (6 isolates). They all contained the $bla_{\text{TEM-61}}$ gene. A polymorphism in the repeat number of a 15-nucleotide sequence (5′-ATGTCATGATAATAA-3′) was found in the promoter region of $bla_{\text{TEM-61}}$; two, three and four repeat units were found in 6, 12 and 2 isolates, respectively. Single nucleotide polymorphism (SNP)-based phylogenetic analysis of 12 isolates revealed that 7 isolates of pulsotype I (12–44 SNP differences) and 5 isolates of pulsotype II (15–55 SNP differences) formed two distinct clusters of genotypes 1 and 2, respectively. All 12 isolates harboured a plasmid carrying the Tn*1*–$bla_{\text{TEM-61}}$ element, although they were slightly different in size (78 883 bp, 78 898 bp and 78 913 bp) owing to differences in the number of 15-bp repetitive sequences. A 42 542-bp broad-host-range plasmid carrying the Tn*1*–$bla_{\text{TEM-61}}$ element was also found in one of the isolates.

Conclusions: We characterised a plasmid-encoded novel Tn*1*–$bla_{\text{TEM-61}}$ element and transposon-dependent mechanisms underlying the propagation of antibiotic resistance, together with repeated new polymorphic 15-bp units in the promoter of $bla_{\text{TEM-61}}$.

1. Introduction

Multidrug-resistant Gram-negative bacteria (MDR-GNB) producing extended-spectrum β-lactamases (ESBLs)/carbapenemases are a major clinical concern in healthcare settings as therapeutic options for serious infections caused by these MDR-GNB are very limited. Multidrug resistance in GNB can be achieved by the accumulation of acquired resistance mechanisms in addition to intrinsic resistance. Mobile genetic elements (MGEs) and/or transposable elements such as epidemic plasmids play a pivotal role in the rapid dissemination of resistance genes among various pathogenic microbes. In transmission events of MDR-GNB in hospital settings, analyses of bacterial phylogeny and MGEs become a very impor-

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tant genomic approach to understand the epidemiology of these organisms.

Serratia marcescens, a ubiquitous environmental bacterium, has been recognised as a relevant opportunistic human pathogen responsible for nosocomial and other severe infections. A major concern of this pathogen showing intrinsic resistance to several antimicrobials is the tendency to spread rapidly in hospital environments, causing outbreaks in high-risk populations and serving as a reservoir contributing to plasmid-mediated dissemination of resistance determinants [1]. Among *S. marcescens* clinical isolates, the CTX-M-3 enzyme has been the most common ESBL type in Poland, Taiwan and Bulgaria and has also been identified in Japan [2–5]. Several reports have documented the involvement of *S. marcescens* producing ESBLs such as CTX-M-3, CTX-M-15, SHV-12 and SHV-5 in hospital outbreaks [6–9]. Overproduction of intrinsic chromosomal AmpC β-lactamase in *S. marcescens* confers resistance to various broad-spectrum cephalosporins except for ceftazidime [10]. TEM-type ESBLs evolved from a narrow-spectrum parent (TEM-1 or TEM-2) are generally more active against ceftazidime than cefotaxime. To date, more than 240 TEM derivatives have been described, of which the majority are ESBLs. TEM-61 (also called 'Cazhi'), having two amino acid substitutions (R164H and E240K) compared with the TEM-2 enzyme, has been reported to show a high level of ceftazidime-hydrolysing activity [11]. To the best of our knowledge, this enzyme has not been reported since its first detection from *Escherichia coli* and *Klebsiella pneumoniae* clinical isolates in Belgium [11,12], and no nucleotide sequence data are available in GenBank. We found that ceftazidime-resistant *S. marcescens* isolates implicated in healthcare-associated infections harboured rare $bla_{\text{TEM-61}}$ genes. Therefore, the aim of this study was to determine the whole DNA sequences of plasmids carrying $bla_{\text{TEM-61}}$ to better understand the spread of resistance mediated by MGEs among *S. marcescens* populations. We identified a $bla_{\text{TEM-61}}$-carrying plasmid specific to *S. marcescens* and transposon-dependent mechanisms for the propagation of antibiotic resistance, and identified a new polymorphic repeat unit in the promoter region of the $bla_{\text{TEM-61}}$ gene.

2. Materials and methods

2.1. Bacterial isolates

A total of 20 non-duplicate (one per patient) ceftazidime-resistant *S. marcescens* clinical isolates implicated in suspected nosocomial transmission were collected from 20 adult patients in a medium-sized hospital with ~300 beds in Japan over the course of a year. The isolates (Sm1 to Sm20) were recovered from 15 respiratory, 4 urine and 1 pus specimen (Table 1). The patients, from seven different wards (predominantly from two main internal medicine wards A and B), comprised 15 males and 5 females with a mean ± standard deviation age of 76 ± 7.8 years.

2.2. Antimicrobial susceptibility testing

Minimum inhibitory concentrations (MICs) were determined by the broth microdilution method using a MicroScan WalkAway 96 Si system (NEG Combo 6.11 J and ESBL plus panels; Beckman Coulter, Tokyo, Japan) and the results were interpreted according to Clinical and Laboratory Standards (CLSI) breakpoints [13]. *Escherichia coli* χ1037 transconjugant with plasmid pSm10-2 carrying $bla_{\text{TEM-61}}$ was also subjected to MIC determination.

2.3. Molecular analysis

All *S. marcescens* isolates were confirmed to be ESBL-producers, harbouring bla_{TEM} ESBL genes as determined by PCR [14]. The nucleotide sequences of the bla_{TEM} genes and their promoter regions were determined by direct sequencing of the PCR products amplified using the primer pair 5′-ATAAAATTCTTGAAGACGAAA-3′ and 5′-GACAGTTACCAATGCTTAATCA-3′. Pulsed-field gel electrophoresis (PFGE) of *Spe*I-digested chromosomal DNA was performed. Broth mating conjugation experiments were carried out to transfer the ceftazidime resistance phenotype of the isolates to *E. coli* χ1037 (Rif^{r}).

2.4. Whole-genome sequencing (WGS)

Twelve selected isolates (Sm1–Sm4, Sm6–Sm8, Sm10, Sm11, Sm14, Sm18 and Sm20) were subjected to WGS [15]. The isolates were selected according to their PFGE pulsotypes and the number of repeat units of a 15-nucleotide sequence in the promoter region of $bla_{\text{TEM-61}}$. Genomic DNA was extracted using a Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA). A library was prepared using an NEBNext® Ultra DNA Library Prep Kit (New England Biolabs, Ipswich, MA, USA), and 150-bp paired-end sequencing was performed using a NovaSeq 6000 platform (Illumina Inc., San Diego, CA, USA). Genome assembly was performed de novo using the A5-miseq pipeline v.20160825. ResFinder 3.2 and PlasmidFinder 2.1 from the Center for Genomic Epidemiology (CGE) (http://www.genomicepidemiology.org) were used for detection of resistance genes and plasmid typing, respectively. Single nucleotide polymorphism (SNP)-based phylogeny was analysed using CGE CSI Phylogeny 1.4 with Sm1 as the reference sequence. Annotation of scaffolds was performed using DFAST (https://dfast.nig.ac.jp/). A whole-genome multilocus sequence typing (wgMLST) tree was constructed using PGAdb-builder (http://wgmlstdb.imst.nsysu.edu.tw). Twenty representative *S. marcescens* genomes obtained from the NCBI (Supplementary Table S1) were included in the wgMLST analysis [16]. The phylogenetic tree was visualised using Interactive Tree of Life (iTOL) v.5 (http://itol.embl.de/). Analysis of drug resistance plasmids was performed manually on WGS data.

2.5. Plasmid sequencing analysis of pSm10-2 carrying $bla_{\text{TEM-61}}$ harboured by Escherichia coli χ1037 transconjugant

Plasmid DNA extracted from *E. coli* χ1037 transconjugant using a PureYield™ Plasmid Midiprep Kit (Promega) was subjected to PFGE, followed by purification with a Wizard® SV Gel and PCR Clean-Up System (Promega). After genomic library construction using a Nextera XT DNA Sample Preparation Kit (Illumina Inc.), sequencing with 150-bp paired-end reads was performed using a MiSeq instrument (Illumina Inc.) [15,17]. De novo assembly into contigs and annotation were performed as described above. The contigs obtained after de novo assembly were subjected to gap-closing PCR and Sanger sequencing to determine the complete nucleotide sequence.

2.6. Nucleotide sequence accession numbers

The whole-genome sequences of the *S. marcescens* isolates sequenced in this study have been deposited in the DDBJ/EMBL/GenBank database under BioProject **PRJNA648651**. The complete nucleotide sequences of plasmids pSm1-1, pSm10-1, pSm10-2, pSm14-1 and pSm14-2 have been deposited at GenBank under accession numbers **LC547239**, **LC545852**, **LC542923**, **LC546831** and **LC542974**, respectively.

3. Results

3.1. Antibiotic susceptibility and molecular characteristics

All 20 *S. marcescens* isolates showed elevated resistance to ceftazidime (MICs, 32 to >128 mg/L), cefpodoxime (MICs, 16 to >64

Table 1
Minimum inhibitory concentrations (MICs) of antimicrobial agents and characteristics of *bla*$_{TEM-61}$-harbouring *Serratia marcescens* isolates

Pulsotype [a]	No. of repeats of a 15-nucleotide sequence [b]	Isolate	Clinical specimen	Hospital ward	MIC (mg/L) [c]										
					CTX	CTX/CLA [d]	CAZ	CAZ/CLA [d]	CRO	CPD	FEP	CMZ	FOX	ATM	IPM
I	2	Sm1	Transtracheal aspirate	A	8	1	>128	0.5	8	>64	8	32	8	>64	≤1
		Sm16	Urine	A	8	4	>128	1	16	>64	>32	≤0.5	4	>64	≤1
		Sm17	Transtracheal aspirate	B	1	0.5	32	≤0.12	1	32	≤1	4	8	32	≤1
	3	Sm5	Nasal discharge	C	4	4	128	0.5	8	>64	4	8	16	>64	2
		Sm7	Sputum	B	4	8	>128	0.5	2	>64	8	8	16	>64	≤1
		Sm8	Transtracheal aspirate	A	1	1	64	0.25	1	32	≤1	8	8	64	≤1
		Sm9	Transtracheal aspirate	A	2	1	32	≤0.12	2	32	≤1	4	8	32	≤1
		Sm10	Transtracheal aspirate	E	8	0.5	>128	0.25	16	>64	32	4	8	>64	≤1
		Sm11	Nasal swab	A	1	1	32	≤0.12	1	32	≤1	8	8	32	≤1
		Sm12	Sputum	D	4	0.5	64	0.5	4	64	4	4	8	32	≤1
		Sm15	Transtracheal aspirate	B	1	0.5	32	0.25	1	16	≤1	8	8	32	≤1
		Sm18	Transtracheal aspirate	B	1	1	32	≤0.12	1	32	2	8	16	32	≤1
	4	Sm14	Transtracheal aspirate	A	8	8	>128	0.5	8	>64	4	16	8	>64	≤1
		Sm19	Transtracheal aspirate	B	1	1	32	≤0.12	1	16	≤1	4	8	32	≤1
II	2	Sm2	Catheter urine	A	8	2	128	0.5	8	>64	4	16	16	>64	2
		Sm4	Pus (foot)	A	8	2	128	0.5	8	>64	4	16	16	>64	≤1
		Sm6	Sputum	A	16	8	128	0.25	8	>64	8	32	16	>64	2
	3	Sm3	Catheter urine	A	8	8	64	1	4	64	2	16	16	64	2
		Sm13	Sputum	G	8	8	64	1	4	32	2	16	32	64	≤1
		Sm20	Urine	F	8	8	128	0.5	>64	>64	8	>32	>32	64	≤1

CTX, cefotaxime; CLA, clavulanic acid; CAZ, ceftazidime; CRO, ceftriaxone; CPD, cefpodoxime; FEP, cefepime; CMZ, cefmetazole; FOX, cefoxitin; ATM, aztreonam; IPM, imipenem.
[a] Isolates differing by up to three fragments were assigned to the same pulsotype.
[b] The 15-nucleotide sequence consisted of 5′-ATGTCATGATAATAA-3′.
[c] MICs of ampicillin, piperacillin and meropenem were >16 mg/L, >64 mg/L and ≤0.5 mg/L, respectively, for all isolates tested.
[d] Clavulanic acid (CLA) at a fixed concentration of 4 mg/L.

mg/L), cefoxitin (MICs, 4 to >32 mg/L) and aztreonam (MICs, 32 to >64 mg/L), while they were susceptible to imipenem (MICs, ≤1–2 mg/L) and meropenem (MICs, ≤0.5 mg/L). For all isolates, the MIC of ceftazidime was decreased drastically (≤0.12–1 mg/L) in the presence of 4 mg/L clavulanic acid (Table 1). PCR and sequencing analyses revealed that all isolates carried the rare *bla*$_{TEM-61}$ ESBL gene. The *bla*$_{TEM-61}$ gene was associated with strong *Pa*/*Pb* promoters similar to TEM-2 [18]. Interestingly, a polymorphic number of repeat units of a 15-nucleotide sequence (5′-ATGTCATGATAATAA-3′) was found in sequences downstream of the *Pb* promoter, with two, three and four repeat units found in 6, 12 and 2 isolates, respectively (Table 1; Fig. 1).

The 20 TEM-61-producing isolates generated two distinct PFGE pulsotypes. Fourteen isolates (Sm1, Sm5, Sm7–Sm12 and Sm14–19) belonged to pulsotype I, while the remaining 6 isolates (Sm2–4, Sm6, Sm13 and Sm20) belonged to pulsotype II. This was found to be irrespective of the hospital ward or the number of repeat units of the 15-nucleotide sequence, with the exception that all five isolates from ward B were of pulsotype I (Table 1).

The isolates were analysed for their ability to transfer ceftazidime resistance to *E. coli*. None of the isolates yielded ceftazidime-resistant transconjugants over multiple experiments, except that a transconjugant with plasmid pSm10-2 carrying *bla*$_{TEM-61}$ was obtained successfully using Sm10 as the donor with a high frequency (2.8×10^{-3} CFU/donor cell). This transconjugant had a ceftazidime MIC of 128 mg/L.

3.2. Assessment of genomic relatedness

SNP-based phylogenetic analysis among 12 TEM-61-producing *S. marcescens* isolates revealed that 7 isolates (Sm1, Sm7, Sm8, Sm10, Sm11, Sm14 and Sm18) in pulsotype I, separated by 12–44 SNPs, were clustered together (genotype 1), while 5 isolates (Sm2–4, Sm6 and Sm20) in pulsotype II, separated by 15–55 SNPs, formed another cluster (genotype 2) (Fig. 2). The genotype 1 isolates were clearly separate from genotype 2 isolates by 22 170–22 209 differing SNPs.

The clonal relationship was assessed by wgMLST gene-by-gene comparison. wgMLST identified two distinct clusters that were concordant with those generated by SNP analysis and corresponded to genotypes 1 and 2. Twelve isolates from genotypes 1 and 2 were closely related to publicly available strains of human clinical origin within the wgMLST tree (Fig. 3).

These 12 isolates carried several antimicrobial resistance genes including the ESBL gene *bla*$_{TEM-61}$, chromosomal *ampC* genes *bla*$_{SRT-2}$ (genotype 1 isolates) or *bla*$_{SRT-2-like}$ (genotype 2 isolates), and the chromosomal aminoglycoside resistance-associated gene *aac(6′)-Ic*. The SRT-2-like enzyme encoded by *bla*$_{SRT-2-like}$ displayed 99.2% amino acid identity (375/378 amino acids) with SRT-2 encoded by *bla*$_{SRT-2}$ (GenBank accession no. **NG_050143**), where the same upstream flanking sequence containing *ampR* (ca. 1000 bp) was shared within each *bla* gene.

3.3. Analyses of *bla*$_{TEM-61}$-associated mobile genetic elements (MGEs)

The complete sequence of a circular plasmid could be determined without filling gaps by PCR from a single plasmid-derived contig containing repeat units of the 15-nucleotide sequence that were positioned internally in the contig. For all 12 representative isolates, the *bla*$_{TEM-61}$ gene was located on a plasmid harbouring 92 protein-coding genes. Due to difference in the number of repeat units of a 15-nucleotide sequence, three different sizes of plasmid were identified though the nucleotide sequences except those repeats were 100% identical among them; 78 883-bp plasmids pSm1-1, 2-1, 4-1 and 6-1 containing two repeating units; 78 898-bp plasmids pSm3-1, 7-1, 8-1, 10-1, 11-1, 18-1 and 20-1 containing three repeating units; and 78 913-bp plasmid pSm14-1 containing four repeating units. These plasmids with non-typeable replicons shared a type II toxin–antitoxin system VapBC and a *tra* cluster of the type IV secretion system connected to the RepA replication region. The *bla*$_{TEM-61}$ gene, carried within a 4964-, 4979- or 4994-bp transposon Tn*1*, was flanked by 5-bp direct repeats (TAAAC) and was situated downstream of the *repA* gene. These 12 plasmids with a 54.0% GC content of plasmid backbone exhibited >95% nucleotide sequence identity and >83% coverage to *S. marcescens*

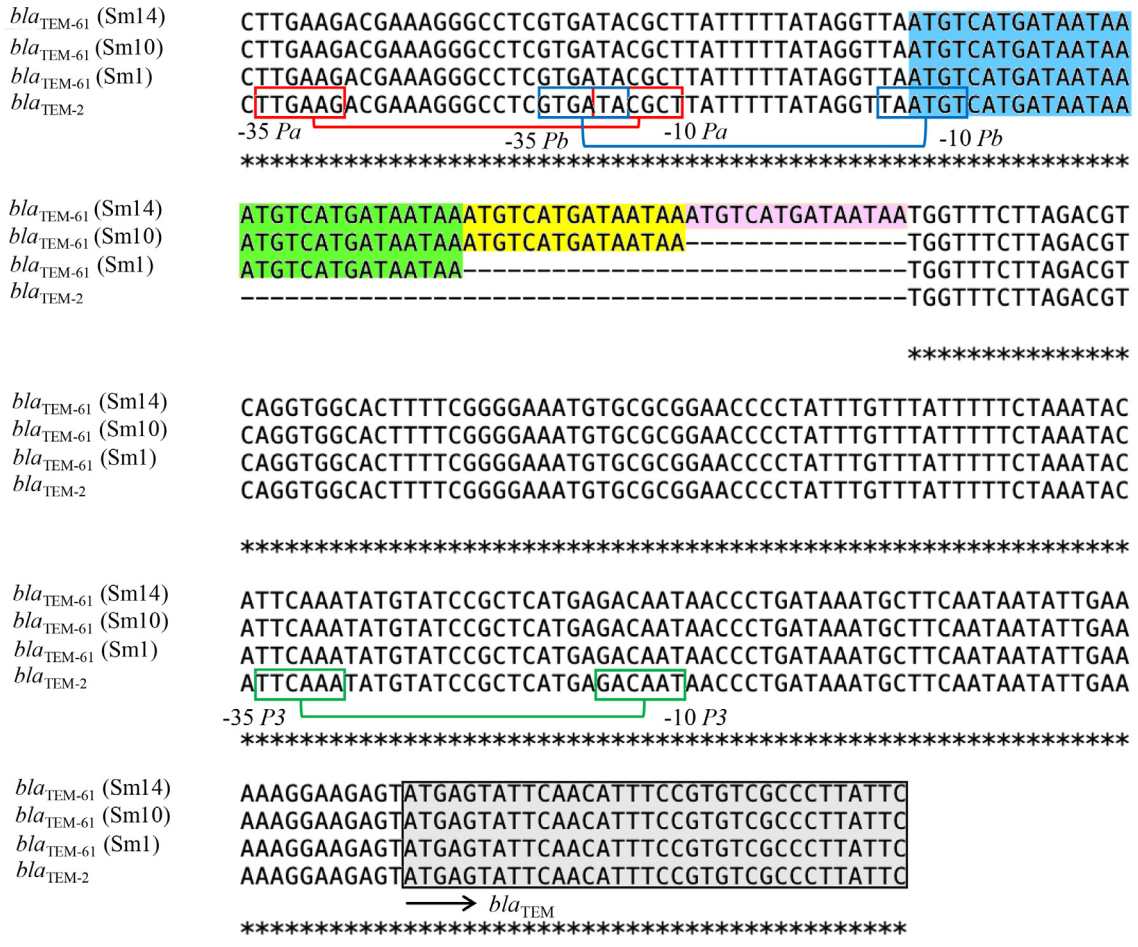


Fig. 1. Nucleotide sequence alignments of representative promoter regions of bla_{TEM-61} genes in *Serratia marcescens* strains Sm1, Sm10 and Sm14 with that of bla_{TEM-2}. The 15-nucleotide direct repeats with no intervening nucleotides differ in the number of repeat units among strains; the first, second, third and fourth units are shaded in blue, green, yellow and pink, respectively. The sequences of the –35 and –10 regions of overlapping promoters *Pa*/*Pb* and a promoter *P3* reported previously [18] are boxed with solid lines in red/blue and green, respectively, and the structural bla_{TEM} genes are shaded.

plasmids not carrying Tn*1*–bla_{TEM-61} transposon insertions discovered from a Singapore air sample (**CP027301**) and nematode cuticle (**LT575491**) (Fig. 4A).

Of note, the 4979-bp Tn*1*–bla_{TEM-61} element [flanked by 5-bp direct repeats (GCTCA)], which was exactly the same as that carried by pSm10-1, was also found in plasmid pSm10-2 harboured by both Sm10 and the *E. coli* χ1037 transconjugant. Complete nucleotide sequence analysis of pSm10-2 revealed it to be a circular plasmid of 42 542 bp in size, with a 61.4% GC content of the plasmid backbone harbouring 47 protein-coding genes. This plasmid with non-typeable replicons had addiction modules and a *trb* cluster of the type IV secretion system as the backbone structure. Remarkably, strain Sm14 had a 37 558-bp plasmid (pSm14-2) that showed 100% nucleotide sequence identity to plasmid pSm10-2, with the exception only of the above 4979-bp Tn*1*–bla_{TEM-61} element that was identified in strain Sm10. pSm14-2 showed >95% nucleotide sequence identity and >92% coverage to plasmids pEC743_4 (**CP015073**), pKPN-704 (**CP014764**) and pJHX613 (**CP020602**) found in *E. coli, K. pneumoniae* and *Pseudomonas aeruginosa*, respectively (Fig. 4B). Such highly transmissible plasmids as pSm10-2 and pSm14-2 were not detected in the other 18 isolates by PCR and WGS (data not shown).

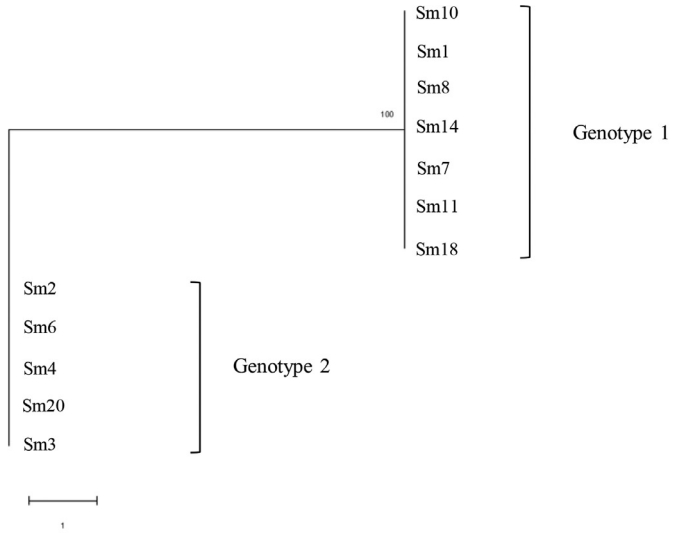
3.4. Virulence-associated genes

Profiles of chromosomally-encoded virulence-associated genes of 12 *S. marcescens* isolates belonging to genotypes 1 and 2 were mostly the same as those of publicly available human clinical strains that clustered together in the wgMLST tree (Fig. 3). These isolates harboured genes involved in haemolysin secretion/activation (*shlB_1, _2* and *_3*) and a contact-dependent growth inhibition (Cdi) system-associated *cdiA* gene, while they were mostly not found among environmental and plant growth-promoting strains.

4. Discussion

In this study, we initially performed analyses of ceftazidime-resistant *S. marcescens* isolates implicated in suspected nosocomial transmission by using conventional molecular methods including PFGE and nucleotide sequencing of PCR products of ESBL genes and their promoter regions, as well as conjugation experiments. All isolates, separated into two pulsotypes, were found to produce a rare TEM-type ESBL (TEM-61), where notably the bla_{TEM-61} gene possessed unique repeated sequences in the promotor region. In repeated experiments, only one isolate generated *E. coli* transconjugants carrying a bla_{TEM-61}-positive plasmid at high frequency. No linkage was expected between these results and the hospital wards or the number of repeated sequences in the bla_{TEM-61} promotor region, which urged us to use a WGS approach to explore the spreading dynamics of the isolates harbouring bla_{TEM-61}.

TEM-61 is reportedly derived from TEM-11 (CAZ-lo) by in vivo selection [11], but *S. marcescens* producing TEM-11 were not detected among nosocomial-related isolates in the present



SNP differences matrix

		Genotype 1							Genotype 2				
		Sm1	Sm7	Sm8	Sm10	Sm11	Sm14	Sm18	Sm2	Sm3	Sm4	Sm6	Sm20
Genotype 1	Sm1	0	26	23	22	25	34	26	22191	22172	22171	22187	22188
	Sm7	26	0	35	34	12	44	13	22191	22172	22171	22187	22188
	Sm8	23	35	0	31	34	43	35	22200	22181	22180	22196	22197
	Sm10	22	34	31	0	33	42	34	22197	22178	22177	22193	22194
	Sm11	25	12	34	33	0	43	7	22190	22171	22170	22186	22187
	Sm14	34	44	43	42	43	0	44	22209	22190	22189	22205	22206
	Sm18	26	13	35	34	7	44	0	22191	22172	22171	22187	22188
Genotype 2	Sm2	22191	22191	22200	22197	22190	22209	22191	0	41	44	24	33
	Sm3	22172	22172	22181	22178	22171	22190	22172	41	0	55	35	36
	Sm4	22171	22171	22180	22177	22170	22189	22171	44	55	0	26	35
	Sm6	22187	22187	22196	22193	22186	22205	22187	24	35	26	0	15
	Sm20	22188	22188	22197	22194	22187	22206	22188	33	36	35	15	0

Fig. 2. Phylogenetic relationships of 12 TEM-61-producing *Serratia marcescens* isolates based on whole-genome single nucleotide polymorphisms (SNP) analysis. A phylogenetic tree was constructed using the distance matrix of SNP differences between each pair of genomes.

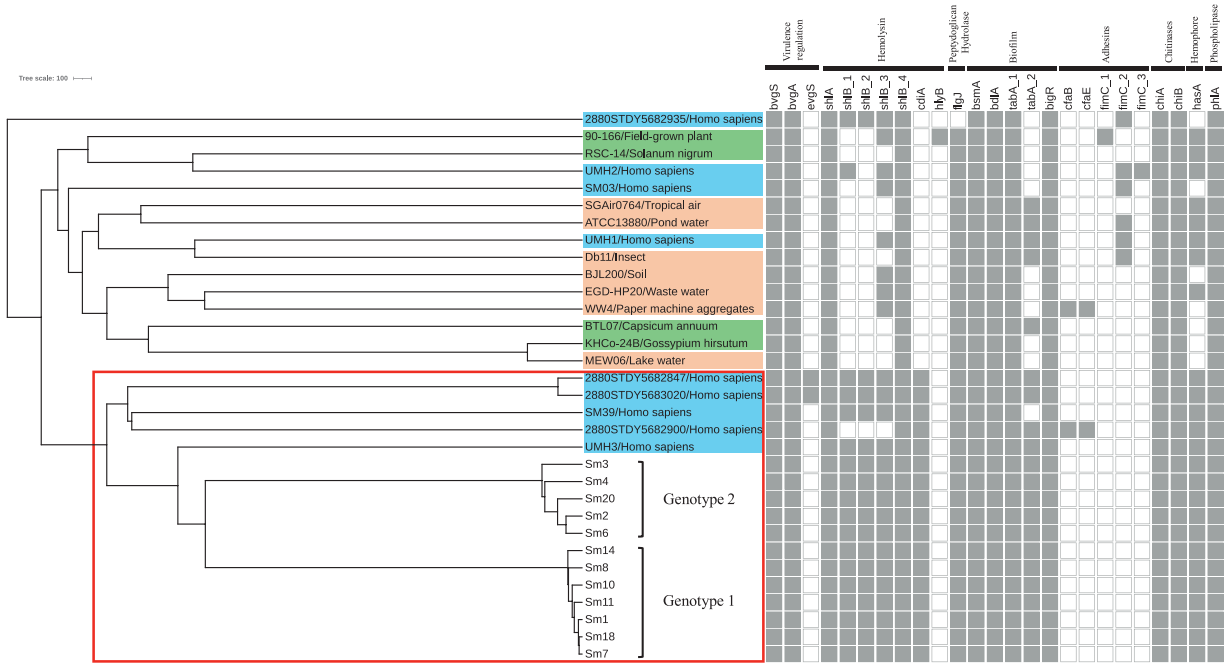


Fig. 3. Whole-genome multilocus sequence typing (wgMLST) tree showing phylogenetic relationships of 12 TEM-61-producing *Serratia marcescens* isolates in this study compared with 20 representative *S. marcescens* strains. The representative strains comprise 9 human clinical (blue), 7 environmental (brown) and 4 plant growth-promoting strains (green). Presence (grey) or absence (white) of 25 virulence-associated genes among strains is shown. The cluster comprising 12 TEM-61-producing isolates (genotypes 1 and 2) and 5 representative strains of human clinical origin is outlined in red.

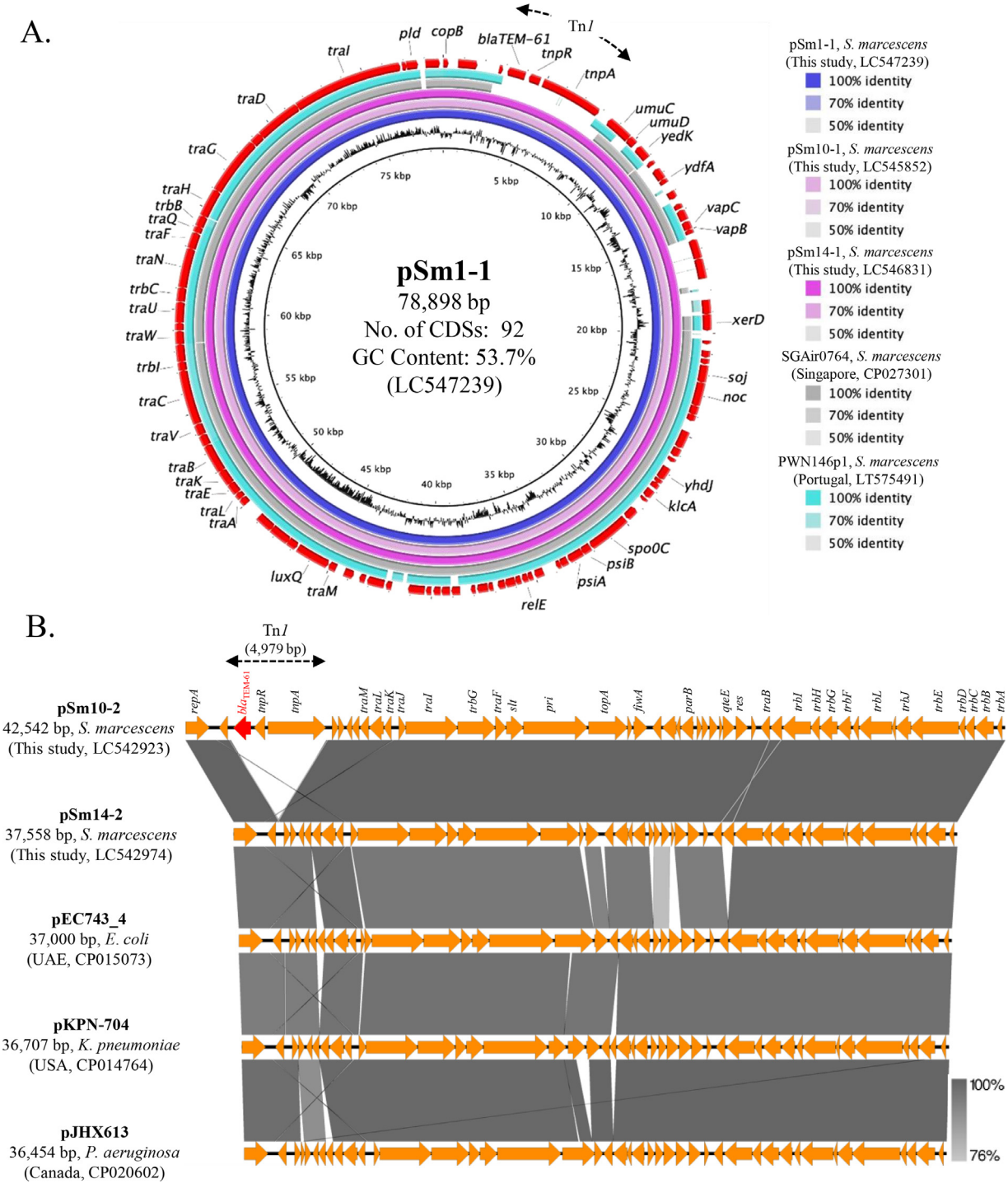


Fig. 4. Genetic comparison of complete plasmid sequences of bla_{TEM-61}-carrying plasmids. (A) Circular comparison between pSm1-1 (accession no. **LC547239**), pSm10-1 (**LC545852**) and pSm14-1 (**LC546831**) in this study and other closely related plasmids SGAir0764 (**CP027301**) and PWN146p1 (**LT575491**). pSm1-1 (innermost, blue), with shared regions with pSm10-1 (pink), pSm14-1 (magenta), SGAir0764 plasmid (grey) and PWN146p1 (turquoise) are depicted using BLAST Ring Image Generator (BRIG) v.0.95 (http://brig.sourceforge.net). The outermost circle shows predicted coding sequences of pSm1-1. (B) Linear comparison of pSm10-2 (**LC542923**) and pSm14-2 (**LC542974**) in this study, pEC743_4 from *Escherichia coli* (**CP015073**), pKPN-704 from *Klebsiella pneumoniae* (**CP014764**) and pJHX613 from *Pseudomonas aeruginosa* (**CP020602**). Alignment of plasmid sequences was generated with Easyfig v.2.2.2 (http://mjsull.github.io/Easyfig/). The arrows show the position and transcriptional direction of open-reading frames (ORFs), and grey shading indicates regions of homology.

study. In all 20 *S. marcescens* isolates, the bla_{TEM-61} gene was associated with the strong overlapping promoters *Pa*/*Pb*, first found upstream of the bla_{TEM-2} gene [18]. It is worth noting that the two, three or four repeat units of a 15-nucleotide sequence of bla_{TEM-2} origin were embedded in sequences just downstream of the –10 region of the *Pb* promoter, generating variations in the numbers of nucleotide residues within the promoter regions. These direct repeat-containing promoter regions may well cause differences in expression of the bla_{TEM-61} gene [19]. These repeating units also contain sequences that could provide putative promoter elements, such as –10 and –35 regions (underlined), adjacent to and within the three repeated units

(TTA ATGTCATGATAATAAATGTCATGATAATAAATGTCATGATAATAA) by in silico prediction using BPROM (http://www.softberry.com/berry.phtml). However, in the present study, there was no apparent difference in the ceftazidime MIC irrespective of the copy numbers of the repeat unit. Whole-genome sequences of 12 isolates showed that amino acid sequences of OmpF, OmpC, PBP1A, PBP1B and PBP2, conferring resistance to β-lactams, were 100% identical among them. Thus, the factors affecting variations in the ceftazidime MIC remain unclear. These unit numbers remained unchanged over repeated subculturing regardless of the presence or absence of ceftazidime as well as in an isolate recovered from the same patient 1 month later (data not shown). We therefore considered that the repeats would be maintained very stably. However, the mechanism of generating these direct repeats, the biological significance of always having more than two repeat units (one-unit sequences originally harboured by Tn*1* are absent) and the microbial meaning of the difference in the number of repeat units should be further explored for better understanding of the diversification process of the genetic context of the *bla* genes.

In several previous outbreak reports of ESBL-producing *S. marcescens*, clonal lineages, transferable/non-transferable plasmids and ESBL genes were identified mainly by PFGE, PCR direct sequencing and conjugation experiments [6–9]. WGS analysis allowed us to identify the involvement of *S. marcescens*-specific plasmids carrying a Tn*1*–$bla_{\text{TEM-61}}$ transposon and two SNP genotypes of isolates in the nosocomial spread of the TEM-61 ESBL in *S. marcescens*. These two genotypes clustered together with public genomes of human clinical origin by wgMLST were genotypically unique in that they shared virulence-associated *shlB* gene clusters (*shlB_1*, *_2* and *_3*) and *cdiA*, which were mostly not found among environmental and plant growth-promoting strains. The *shlBA* operon encodes a two-partner type V secretion system, where *shlB* encodes an outer membrane protein belonging to the Omp85 subfamily that is essential for secretion/activation of the ShlA haemolysin. The ShlA haemolysin is one of the major virulence factors of *Serratia* spp. causing haemolysis of human erythrocytes and the release of inflammatory mediators from leukocytes [20]. Major mechanisms responsible for the spread of Tn*1*–$bla_{\text{TEM-61}}$ transposons and plasmids carrying them may include the spread of host–plasmid combination and horizontal plasmid transfer between genotypes 1 and 2. Moreover, strain Sm10 was found to have two different plasmids carrying the Tn*1*–$bla_{\text{TEM-61}}$ transposon, suggesting a replicative transposition event. This may be supported by the finding that strain Sm14 also had the same combinations of plasmids, one carrying the Tn*1*–$bla_{\text{TEM-61}}$ transposon and the other not. The intracellular translocation of Tn*1*–$bla_{\text{TEM-61}}$ from a narrow-host-range plasmid that has been identified only in *S. marcescens* to a broad-host-range plasmid that has been harboured by *E. coli, K. pneumoniae* and *P. aeruginosa* (Fig. 4B) is a problem of concern due to the high transmission potential of the narrow-host-range plasmid carrying $bla_{\text{TEM-61}}$ gene to other bacterial species.

In conclusion, we report the transmission dynamics of a quite rare ESBL gene ($bla_{\text{TEM-61}}$) via a MGE among two genotypes of isolates during nosocomial spread of ceftazidime-resistant *S. marcescens*. Our findings show that Tn*1*–$bla_{\text{TEM-61}}$ is strategically transposed from a *S. marcescens*-specific plasmid to a broad-host-range plasmid that has a high transfer frequency. We identified that the $bla_{\text{TEM-61}}$ gene has different units of direct repeats with no intervening nucleotides in its promoter region. This study highlights the importance of tracking the movement of MGEs that carry resistance genes among the MDR-GNB associated with nosocomial infections. This may enable us to estimate the true extent of the circulation of resistance-associated MGEs in a hospital setting. Further studies would be required to investigate the general prevalence of the $bla_{\text{TEM-61}}$ gene among GNB in other healthcare settings in order to explore the spreading mechanisms of this ESBL gene.

Declaration of Competing Interests

None declared.

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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.2021.03.010.

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