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Clonal Dissemination of *Pseudomonas aeruginosa* Sequence Type 235 Isolates Carrying bla_{IMP-6} and Emergence of bla_{GES-24} and bla_{IMP-10} on Novel Genomic Islands PAGI-15 and -16 in South Korea

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A total of 431 *Pseudomonas aeruginosa* clinical isolates were collected from 29 general hospitals in South Korea in 2015. Antimicrobial susceptibility was tested by the disk diffusion method, and MICs of carbapenems were determined by the agar dilution method. Carbapenemase genes were amplified by PCR and sequenced, and the structures of class 1 integrons surrounding the carbapenemase gene cassettes were analyzed by PCR mapping. Multilocus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE) were performed for strain typing. Whole-genome sequencing was carried out to analyze *P. aeruginosa* genomic islands (PAGIs) carrying the bla_{IMP-6}, bla_{IMP-10}, and bla_{GES-24} genes. The rates of carbapenem-nonsusceptible and carbapenemase-producing *P. aeruginosa* isolates were 34.3% (148/431) and 9.5% (41/431), respectively. IMP-6 was the most prevalent carbapenemase type, followed by VIM-2, IMP-10, and GES-24. All carbapenemase genes were located on class 1 integrons of 6 different types on the chromosome. All isolates harboring carbapenemase genes exhibited genetic relatedness by PFGE (similarity > 80%); moreover, all isolates were identified as sequence type 235 (ST235), with the exception of two ST244 isolates by MLST. The bla_{IMP-6}, bla_{IMP-10}, and bla_{GES-24} genes were found to be located on two novel PAGIs, designated PAGI-15 and PAGI-16. Our data support the clonal spread of an IMP-6-producing *P. aeruginosa* ST235 strain, and the emergence of IMP-10 and GES-24 demonstrates the diversification of carbapenemases in *P. aeruginosa* in Korea.

Pseudomonas aeruginosa is an opportunistic pathogen that causes various nosocomial infections, including sepsis, pneumonia, and urinary tract infections (1). Treatment of *P. aeruginosa* infections is often difficult because of the intrinsic drug resistance of *P. aeruginosa* and the ability of this pathogen to acquire genes for antimicrobial resistance determinants (2). Carbapenems are used as last-resort drugs for the treatment of infections caused by multidrug-resistant *P. aeruginosa*, due to their high affinity for penicillin-binding proteins, stability to various β-lactamases, and ability to easily pass through the bacterial outer membrane. However, the increasing use of carbapenems has resulted in the emerging phenomenon of carbapenem resistance (3).

P. aeruginosa can become resistant to carbapenems by reduced permeability of the outer membrane due to loss of substrate-specific outer membrane porin OprD, frequently accompanied by AmpC hyperproduction and overexpression of efflux systems (4), along with acquisition of genes encoding carbapenemases (5). While the molecular mechanism of carbapenem resistance is geographically variable, diverse classes of carbapenemase have been increasingly identified in *P. aeruginosa*, including class A (KPC and GES variants), class B (IMP, VIM, and NDM metallo-β-lactamases [MBLs]), and class D (OXA variants) (6). The MBLs, in particular IMP and VIM enzymes, are the most widespread carbapenemases in *P. aeruginosa* (7), with IMP-6 exclusively present in South Korea (8).

Carbapenemase genes are found often on integrons in *P. aeruginosa* clinical isolates. Integrons are prevalent in Gram-negative clinical isolates and offer the ability to capture and excise gene cassettes, frequently including antibiotic resistance determinants, to the bacterial host (9). However, integrons accomplish mobility only when they are associated with a specific mobile genetic element, such as a transposon, conjugative plasmid, or genomic island (10). The genomic islands in *P. aeruginosa* are mostly integrative and conjugative elements (ICEs) and are named according to their characteristics, i.e., *P. aeruginosa* pathogenicity island or the name of their host, i.e., Liverpool epidemic strain genomic islands or, more broadly, i.e., *P. aeruginosa* genomic island (PAGI) (11). Thus far, 14 PAGIs have been identified (12). These genomic islands typically carry genes for integration, transfer, and maintenance, which confer self-transferability to the composite ICE, along with genes conferring metabolic, virulence, and heavy metal resistance capability, which offer extra benefit to the bacterial host. Although PAGIs associated with antimicrobial resistance are rare, some examples have been identified, e.g., derivatives of PAGI-1 and PAGI-2 (13).

Two nucleotide sequence-based bacterial typing techniques, pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST), are considered to be the gold standards for use in epidemiological studies. The PFGE provides fingerprints of entire bacterial genomes by restriction enzyme mapping to determine

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epidemiological relationships of an outbreak in detail, while the MLST method gives strain lineages based on DNA fragments of seven housekeeping genes which allow fewer and larger groups feasible for global comparison (14). Two international high-risk *P. aeruginosa* clones, sequence type 111 (ST111) and ST235, are responsible for the dissemination of carbapenemase genes worldwide (15), with GES-type carbapenemase-producing *P. aeruginosa* ST235 in Spain (16) and IMP-6-producing *P. aeruginosa* ST235 in South Korea (8). Meanwhile, ST175 and ST244 carrying genes that confer resistance to carbapenems have also been reported to be responsible for regional dissemination; as a result, it could be considered that they were associated with high-risk clones (17). Together with PFGE and MLST, relatively high-cost whole-genome sequencing (WGS) is now commonly used for epidemiological studies expecting an incomparable power of discrimination (14).

The aims of this study were to investigate the prevalence of carbapenemase genes in *P. aeruginosa* clinical isolates recovered from general hospitals in South Korea in 2015 and to determine the genetic environments surrounding the carbapenemase genes and epidemiological characteristics of carbapenemase-producing *P. aeruginosa* (CP-PA) clinical isolates.

MATERIALS AND METHODS

Bacterial strains. A total of 431 nonduplicated *P. aeruginosa* clinical isolates were collected from 29 general hospitals located in 11 cities and provinces in South Korea between June and August 2015. The isolates were recovered from blood ($n = 33$), respiratory specimens ($n = 180$), urine ($n = 93$), pus ($n = 97$), and other specimens ($n = 28$). Bacterial species were identified by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI MS) using a Bruker MALDI MS instrument (Bruker, Billerica, MA). Species identification was made only when the log score of the species was above 2.0; otherwise, partial sequences of the 16S rRNA gene were analyzed.

Antimicrobial susceptibility testing. Antimicrobial susceptibilities were tested by the disk diffusion method by following the Clinical and Laboratory Standards Institute guidelines (18). Briefly, disks (Oxoid Ltd., Basingstoke, UK) containing the following antimicrobial agents were used: piperacillin, ceftazidime, cefepime, aztreonam, gentamicin, tobramycin, amikacin, ciprofloxacin, meropenem, imipenem, and colistin. The MICs of meropenem and imipenem were determined by the agar dilution method on Mueller-Hinton agar (Becton, Dickinson and Company, Sparks, MD). *P. aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922 were used as control strains.

Identification of carbapenem resistance determinants. The genes encoding IMP-, VIM-, and NDM-type MBLs, KPC- and GES-like serine β-lactamases (19), and OprD outer membrane porin were amplified by PCR in carbapenem-nonsusceptible *P. aeruginosa* clinical isolates. PCR amplicons were purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany) and sequenced using an Applied Biosystems 3730xl DNA analyzer (Applied Biosystems, Foster City, CA) to identify the variant types of each carbapenemase family. Sequences were compared with those in GenBank (http://www.ncbi.nlm.nih.gov/GenBank) using the BLAST network service.

Integron mapping and sequencing. The structures of the class 1 integrons carrying the carbapenemase gene cassettes were determined by PCR mapping and sequencing using the primers listed in Table 1.

MLST. PCR amplification and sequencing of partial fragments of seven housekeeping genes (*acsA*, *aroE*, *guaA*, *mutL*, *nuoD*, *ppsA*, and *trpE*) were performed, and the experimentally determined nucleotide sequences of both strands were compared to preexisting sequences in the MLST database to assign allele numbers and STs (http://pubmlst.org/paeruginosa).

PFGE. Agarose plugs containing XbaI-digested genomic DNA from *P. aeruginosa* clinical isolates were prepared and DNA fragments were separated for 20 h at 6 V/cm at 11°C using a CHEF-DRII system (Bio-Rad, Hercules, CA). Pulsing was carried out with initial and final pulse times of 0.5 s and 30 s, respectively. A lambda ladder (Bio-Rad) was used as a DNA size marker. Band patterns were analyzed with UVIband/Map software (UVItech Ltd., Cambridge, UK) and used to generate dendrograms based on the unweighted pair group method using arithmetic averages from the Dice coefficient.

Southern blot and hybridization. Southern blotting was performed to determine the locations of the carbapenemase genes. Briefly, I-*Ceu*I or S1 nuclease-digested DNA was blotted onto nylon membranes (Zeta-Probe blotting membranes; Bio-Rad) and hybridized with probes specific for the carbapenemase genes or 16S rRNA. The probes were obtained via PCR amplification as described above. Probe labeling, hybridization, and detection were performed with a digoxigenin DNA labeling and detection kit (Roche Diagnostics, Indianapolis, IN).

WGS. For bacterial whole-genome sequencing (WGS), single-molecule real-time (SMRT) sequencing was carried out on a PacBio RSII instrument (Pacific Biosciences, Menlo Park, CA). Genomic DNA was extracted from the *P. aeruginosa* strains using a Wizard genomic DNA purification kit (Promega, Madison, WI). After extraction, DNA shearing was performed using a g-TUBE apparatus (Covaris, Inc., Woburn, MA), and the fragments were purified by using 0.45× the final volume of washed Agencourt AMPure XP magnetic beads (Beckman Coulter Inc., Brea, CA). SMRTbell template libraries were subsequently prepared, and adapter ligation was performed, followed by exonuclease digestion of incompletely ligated products. Reads with lengths that were less than 50 bp were filtered out after acquisition of the sequencing data; the minimum polymerase read quality was set to 0.75. *De novo* genome assembly of PacBio SMRT reads was performed with the PacBio SMRT analysis software suite (version 2.3.0) (20). Briefly, the hierarchical genome assembly process was used with default parameters and a seed read length cutoff of 6 kb. Following assembly, individual contigs with duplicate sequences on their 5′ and 3′ ends were manually trimmed. Similarly, overlapping sequences were manually trimmed and joined based on sequence similarity to form circular fragments. Following chromosome and plasmid circularization, the sequences were polished using Quiver. In this program, the raw reads are mapped back to the chromosome and plasmid sequences to validate the assembly and resolve any remaining sequence errors. The annotations of coding sequences, tRNA sequences, and rRNA sequences were performed using the NCBI Prokaryotic Genome Annotation Pipeline (http://www.ncbi.nlm.nih.gov/books/NBK174280).

Accession number(s). The nucleotide sequence data reported in this paper are available in the GenBank nucleotide database under accession numbers KX196167 (PAGI-16 carrying the $bla_{\text{IMP-6}}$ gene), KX196168 (PAGI-15 carrying the $bla_{\text{GES-24}}$ gene), and KX196169 (PAGI-16 carrying the $bla_{\text{IMP-10}}$ gene).

RESULTS

Antimicrobial resistance profiles of the *P. aeruginosa* isolates. Of the 431 *P. aeruginosa* clinical isolates examined, 283 (65.7%) were found to be susceptible to carbapenems. The remaining 148 isolates (34.3%) that were nonsusceptible to imipenem and/or meropenem were classified by PCR into two groups: carbapenemase-producing *P. aeruginosa* (CP-PA) ($n = 41$), whose members carried the bla_{IMP}, bla_{VIM}, or bla_{GES} gene, and non-carbapenemase-producing *P. aeruginosa* (NCP-PA; $n = 107$), whose members did not. Twenty isolates (18.7%) in the NCP-PA group had an OprD porin loss. The vast majority (40/41) of the clinical isolates in the CP-PA group exhibited an extensively drug-resistant phenotype and were resistant to all drugs tested except colistin (Fig. 1). Moreover, the CP-PA group showed extremely high rates of nonsusceptibility to piperacillin (97.6%, compared to 62.6% in the NCP-PA group and 10.2% in the carbapenem-susceptible group), ceftazidime (97.6%, compared to 29.9% and

TABLE 1 Primers used in this study for integron characterization

Target	Primer	Nucleotide sequence (5′–3′)	Position[a]	Reference
intI	INT1-mF	GCCTGTTCGGTTCGTAAGCT	908/927	This study
	INT1-F	CCAAGCTCTCGGGTAACATC	462/481	
	INT1-R	CATGAAAACCGCCACTGC	997/1014	
qacEΔ1	qacEΔ1-F	GAAAGGCTGGCTTTTTCTTG	3/22	This study
	qacEΔ1-R	GCAGCGACTTCCACGATG	311/328	
ereA	ereA2-F	TTGAGCGATTTTCGGATACC	269/288	This study
	ereA2-R	GGCATGAATCCTCCTTACCA	1071/1090	
qac	qac-F	CAATCTTTGGCGAGGTCATC	29/48	8
	qac-R	CGCTGACCTTGGATAGCAG	307/325	
catB3	catB3-F	AAGGCAAGCTGCTTTCTGAG	29/48	21
	catB3-R	AACGATAGCGTAAGGCTCCA	440/459	
aadA1	aadA1-F	ACATCATTCCGTGGCGTTAT	284/303	This study
	aadA1-R	AGGTTTCATTTAGCGCCTCA	489/508	
aacA4	aacA4-F	TGACCTTGCGATGCTCTATG	12/31	This study
	aacA4-R	CTGGCGTGTTTGAACCATGT	470/489	
aacA7	aacA7-F	CAGGCCTGTTGAAACTACCG	21/40	22
	aacA7-R	CTTGAGCAACCTCCGTGAAT	414/433	
bla_{OXA}	OXA-1F	TATCTACAGCAGCGCCAGTG	54/73	21
	OXA-1R	TGCACCAGTTTTCCCATACA	635/654	
	OXA-2F	CGATAGTTGTGGCAGACGAA	128/147	
	OXA-2R	CTTGACCAAGCGCTGATGT	564/582	
tniC	TniC-R	TTTCCGAGCGAACAGTCGCT	229/248	22
bla_{IMP}	IMP-1F	AAGGCGTTTATGTTCATACTTCG	95/117	19
	IMP-1R	TTTAACCGCCTGCTCTAATGTAA	677/699	
bla_{VIM}	VIM-2F	ATCATGGCTATTGCGAGTCC	46/65	19
	VIM-2R	ACGACTGAGCGATTTGTGTG	775/794	
bla_{KPC}	KPC-F	GTCACTGTATCGCCGTCTAGTTC	3/25	19
	KPC-R	TGGTGGGCCAATAGATGATT	919/938	
bla_{GES}	GES-F	ATGCGCTTCATTCACGCAC	1/19	This study
	GES-R	CTATTTGTCCGTGCTCAGGA	864/845	
bla_{NDM}	NDM-F	GCCCAATATTATGCACCCGG	9/28	19
	NDM-R	CTCATCACGATCATGCTGGC	649/668	
oprD	oprD-F	GGAACCTCAACTATCGCCAAG	−120/−99	This study
	oprD-R	GTTGCCTGTCGGTCGATTAC	17/1328	

[a] Coordinates refer to the first base of each gene.

7.1%, respectively), ciprofloxacin (100%, compared to 70.1% and 13.8%, respectively), and amikacin (100%, compared to 23.4% and 3.5%, respectively).

Presence of the carbapenemase gene and its correlation with carbapenem susceptibility. The most prevalent gene, bla_{IMP-6}, was identified in 36 isolates; moreover, another subtype, bla_{IMP-10}, was identified in one isolate. *P. aeruginosa* clinical isolates harboring bla_{IMP-6} exhibited higher resistance to meropenem (MICs, 64 to >256 μg/ml; MIC_{50}, >256 μg/ml; and MIC_{90}, >256 μg/ml) than to imipenem (MICs, 16 to 256 μg/ml; MIC_{50}, 16 μg/ml; and MIC_{90}, 32 μg/ml). Isolate BP14, carrying bla_{IMP-10}, had similar carbapenem MICs: for meropenem, >256 μg/ml, and for imipenem, 16 μg/ml. The products encoded by bla_{IMP-6} and bla_{IMP-1} differ by one amino acid, with the former having a Ser216-Gly substitution compared with the latter. Similarly, the product encoded by bla_{IMP-10} exhibits a single Val49-Phe substitution compared with the product encoded by bla_{IMP-1}.

The bla_{VIM-2} gene was identified in three isolates. These three isolates had meropenem MICs ranging from 16 to 128 μg/ml and imipenem MICs ranging from 16 to 256 μg/ml. The bla_{GES-24} gene was identified in one isolate, and the isolate showed resistance to meropenem (MIC, 128 μg/ml) and imipenem (MIC, 64 μg/ml).

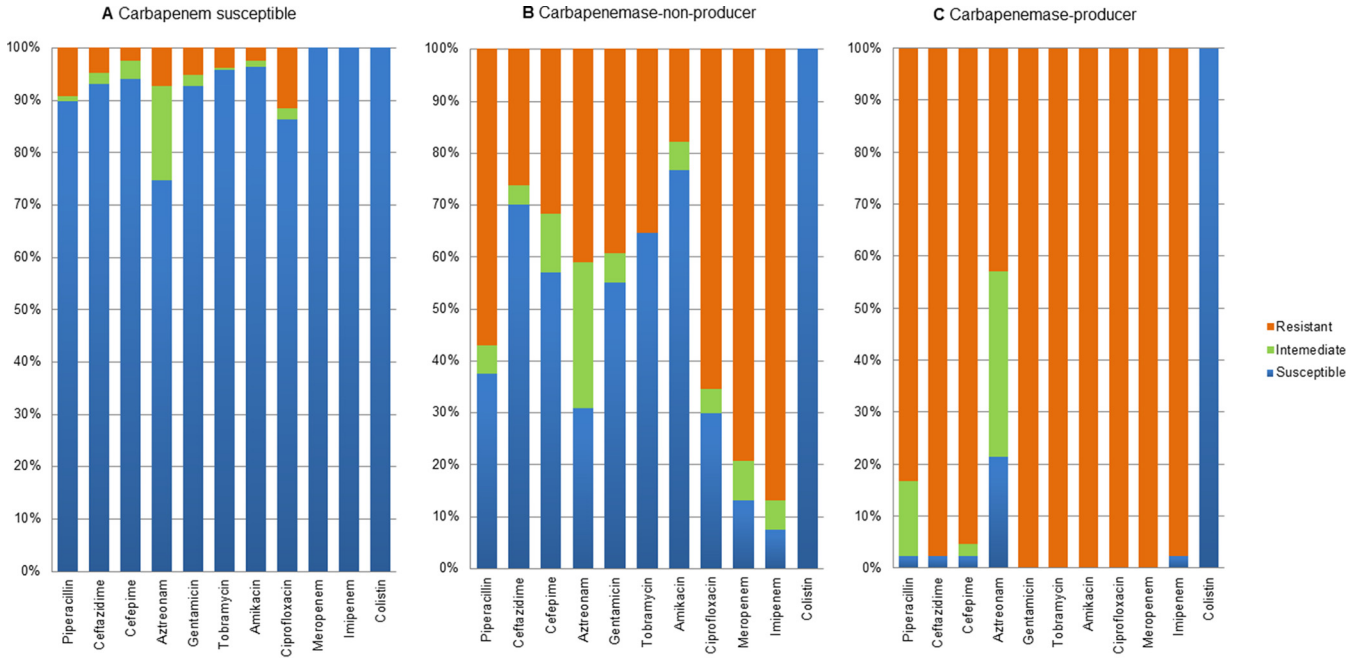


FIG 1 Antimicrobial susceptibilities of *P. aeruginosa* clinical isolates. (A) Carbapenem-susceptible isolates ($n = 283$); (B) carbapenem-nonsusceptible non-carbapenemase producers ($n = 107$); (C) carbapenem-nonsusceptible carbapenemase producers ($n = 41$).

Of note, this strain had a truncated OprD by a novel insertion sequence, IS*PA67*. Considering the relatively low carbapenem MICs for 20 *P. aeruginosa* strains having only the porin loss, 1 to 32 μg/ml (MIC_{50}, 8 μg/ml, and MIC_{90}, 16 μg/ml) for meropenem and 4 to 32 μg/ml (MIC_{50}, 16 μg/ml, and MIC_{90}, 32 μg/ml) for imipenem, the elevated carbapenem MICs were mostly by GES-24. GES-24 differs from GES-1 by two substitutions, Met62-Thr and Gly170-Ser, and the latter change is the same as in the carbapenem-hydrolyzing GES-5.

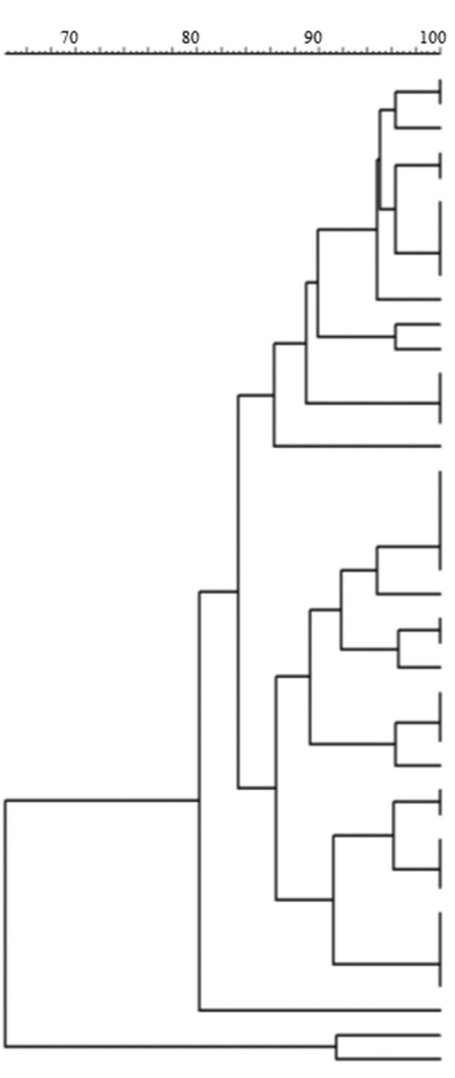
Strain typing for the CP-PA isolates. All CP-PA isolates were identified as ST235 (allele profile, 38-11-3-13-1-2-4), with the exception of two isolates: isolate JNH6, which carried bla_{VIM-2}, and isolate IH2, which carried bla_{GES-24}. These two isolates were identified as ST244 (17-5-12-3-14-4-7), which is very different from ST235 (see Table S1 in the supplemental material). The ST235 CP-PA isolates shared more than 80% similarity as determined by PFGE analysis, and the remaining two ST244 isolates also exhibited close relatedness (similarity, >90%). The ST235 and ST244 isolates did not show significant relatedness (similarity, <70%) to each other (Fig. 2).

Genetic contexts of the class 1 integrons. The bla_{IMP-6}, bla_{IMP-10}, bla_{VIM-2}, and bla_{GES-24} genes were located on class 1 integrons as gene cassettes, and the structures determined by PCR mapping are indicated in Fig. 3. The bla_{IMP-6} gene in all 36 isolates was carried by InIMP-6D (*intI1-*bla_{IMP-6}*-qac-aacA4-catB3-aacA4-*bla_{OXA-1}*-aadA1*). This class 1 integron was identified in 2008 in one *P. aeruginosa* ST235 clinical isolate from South Korea (21). The bla_{IMP-10} gene in isolate BP14 composed a new class 1 integron, InIMP-10 (*intI1-*bla_{IMP-10}*-qac-aacA4-catB3-aacA4-*bla_{OXA-1}*-aadA1*). Between the integron integrase coding gene and the gene cassettes, an integron-associated 136-bp *attI* recombination site was identified.

The bla_{VIM-2} gene cassette was found in three different integrons: In559 (*intI1-aacA7-*bla_{VIM-2}*-dhfa-aadA5*), InVIM-2_{JH5} (*intI1-aadB-*bla_{VIM-2}*-aacA4-orf-ereA*), and InVIM-2_{JH6} (*intI1-aacA4-*bla_{VIM-2}*-aacA4-fosC-*bla_{OXA-2}*-qac*). In559 was first identified in *P. aeruginosa* ST235 clinical isolates from Russia in 2002 (22), and InVIM-2_{JH5} and InVIM-2_{JH6} class 1 integrons were first found in *P. aeruginosa* and *Pseudomonas putida* isolates, respectively, from South Korea (unpublished data; GenBank accession numbers EF207719 for InVIM-2_{JH5} and AY907717 for InVIM-2_{JH6}). Notably, In559 was distinct from the other class 1 integrons that culminate with *qac*EΔ*1* in that the Tn*5090 tniC* gene (also called *tniR* of Tn*402*) for the 3′ conserved sequence (CS) indicates a distinct evolutionary path driving the excision and addition of gene cassettes (23). Isolate IH2 was found to harbor a novel integron, InGES-24 (*intI1-aacA4-aadB-*bla_{GES-24}*-aacA4-*bla_{OXA-2}).

Two novel genomic islands, PAGI-15 and PAGI-16. By Southern blotting using I-*Ceu*I macrorestriction-digested fragments, the bla_{IMP-6}, bla_{IMP-10}, bla_{VIM-2}, and bla_{GES-24} genes were verified to be chromosome carried in all CP-PA isolates (data not shown). Probes specific for the carbapenemase genes did not bind to any S1 nuclease-treated plasmids. To further characterize the genomic environments of the class 1 integrons, WGS was conducted using the PacBio RSII platform for three *P. aeruginosa* isolates: KMU11 of bla_{IMP-6}, BP14 of bla_{IMP-10}, and IH2 of bla_{GES-24}.

The draft genome sequences of KMU11 were composed of nine contigs, and three (a total of 6.9 Mb in size) of those carried the genes for ribosomal proteins indicating that they were carried on a chromosome. The class 1 integron InIMP-6D was found on a chromosome in a 1.6-Mb contig possessing together the genes encoding ribosomal proteins S6, S9, S15, S18, S20, L9, L13, L21, L25, L27, and L31. Moreover, the integron was in a novel genomic island, PAGI-16, which was 95,029 bp long. PAGI-16 was inte-



PFGE patterns	Isolate	Carbapenemase gene	MIC (μg/ml)		Sequence type
			Meropenem	Imipenem	
	CMDAE1	bla_{IMP-6}	>256	32	ST235
	SCHGM14	bla_{IMP-6}	>256	16	ST235
	CMDAE3	bla_{IMP-6}	>256	16	ST235
	CMDAE6	bla_{IMP-6}	>256	16	ST235
	CMDAE7	bla_{IMP-6}	>256	16	ST235
	CBU1	bla_{IMP-6}	>256	16	ST235
	CBU14	bla_{IMP-6}	>256	16	ST235
	CMDAE11	bla_{IMP-6}	>256	16	ST235
	CMDAE12	bla_{IMP-6}	>256	16	ST235
	CMDAE4	bla_{IMP-6}	>256	16	ST235
	CMDAE5	bla_{IMP-6}	>256	16	ST235
	SCHGM15	bla_{IMP-6}	64	16	ST235
	GS4	bla_{VIM-2}	128	256	ST235
	KMU11	**bla_{IMP-8}**	**>256**	**32**	**ST235**
	IH6	bla_{IMP-6}	>256	16	ST235
	KMU3	bla_{IMP-6}	>256	32	ST235
	BP13	bla_{IMP-6}	>256	256	ST235
	CSU11	bla_{IMP-6}	>256	16	ST235
	KMU4	bla_{IMP-6}	>256	32	ST235
	SCHGM1	bla_{IMP-6}	>256	16	ST235
	SCHBU12	bla_{IMP-6}	>256	32	ST235
	JNH1	bla_{IMP-6}	>256	32	ST235
	BP14	**bla_{IMP-10}**	**>256**	**16**	**ST235**
	BP7	bla_{IMP-6}	>256	16	ST235
	CMDAE15	bla_{IMP-6}	>256	16	ST235
	SCHGM10	bla_{IMP-6}	>256	16	ST235
	SCHGM13	bla_{IMP-6}	>256	16	ST235
	SCHGM12	bla_{IMP-6}	>256	16	ST235
	CAU11	bla_{IMP-6}	>256	16	ST235
	GNU11	bla_{IMP-6}	>256	16	ST235
	GNU14	bla_{IMP-6}	>256	16	ST235
	CMDAE14	bla_{IMP-6}	>256	16	ST235
	SCHGM4	bla_{IMP-6}	>256	32	ST235
	WCH1	bla_{IMP-6}	>256	16	ST235
	CSU6	bla_{IMP-6}	>256	32	ST235
	JN5	bla_{IMP-6}	>256	64	ST235
	EUMC2	bla_{IMP-6}	>256	32	ST235
	HP6	bla_{IMP-6}	>256	32	ST235
	JNH5	bla_{VIM-2}	32	16	ST235
	IH2	**bla_{GES-24}**	**128**	**64**	**ST244**
	JNH6	bla_{VIM-2}	16	128	ST244

FIG 2 Characteristics of chromosome-encoded carbapenemase-producing *P. aeruginosa* clinical isolates. Strains which were subjected to whole-genome sequencing are shown in bold.

grated at the end of the $tRNA^{Gly}$ gene, like the other genomic islands found in *P. aeruginosa* (11), and flanked at its end by 20-bp direct repeats (DR), GATTCCCTTCACCCGCTCCA (Fig. 4A). PAGI-16 in isolate KMU11 had a G+C content of 61.4% and contained 99 open reading frames (ORFs), including a cluster of nine genes originating from the *clc* element, which is also found in PFGI-1 of *Pseudomonas fluorescens* (24). The *clc* element integrates through the *attB* site using a bacteriophage P4-like integrase; this site is situated in between two tandem $tRNA^{Gly}$ genes in the 3′ end (11). The cluster also contains 10 genes associated with genome instability, such as genes for integrases and transposases and insertion sequences; eight genes for antimicrobial resistance, i.e., two copies of *aacA4* plus *aadA1*, *catB3*, *cmx*, *sul1*, and bla_{OXA-1}, together with bla_{IMP-6}, which are mostly found in the class 1 integron between bp 57464 and 77106; and genes encoding hypothetical proteins, which comprise the rest of the sequences.

Isolate BP14 had a 7.1-Mb circularized chromosome without any plasmid, and the class 1 integron InIMP-10 was found within a 69,208-bp 5′ portion of a genomic island sharing 99.99% nucleic acid identity with PAGI-16 in isolate KMU11. This portion was integrated at the end of the $tRNA^{Gly}$ gene, and the 20-bp DR GATTCCCTTCACCCGCTCCA was present in the 5′ end as in KMU11. PAGI-16 was ended by IS*6100*, and a truncated *wspC*, encoding a biofilm formation methyltransferase WspC, was found downstream (Fig. 4A). The 1,269-bp *wspC* gene encodes the biofilm formation methyltransferase WspC, and the left 619 bp was found 3.3 Mb away from the portion together with the rest of PAGI-16 in the opposite direction. The 26,701-bp 3′ portion of PAGI-16 in isolate BP16 was identical to that in KMU11, and the 20-bp DR sequence was found, indicating chromosomal inversion by a duplication-insertion of IS*6100* (25). All of PAGI-16 in isolate BP14 had the same G+C content, 61.4%.

IH2 had a 7.3-Mb circularized chromosome devoid of any plasmid. In the chromosome, the class 1 integron InGES-24 was located within a 118,715-bp genomic island designated PAGI-15. The location of PAGI-15 was the same as that of PAGI-16, next to

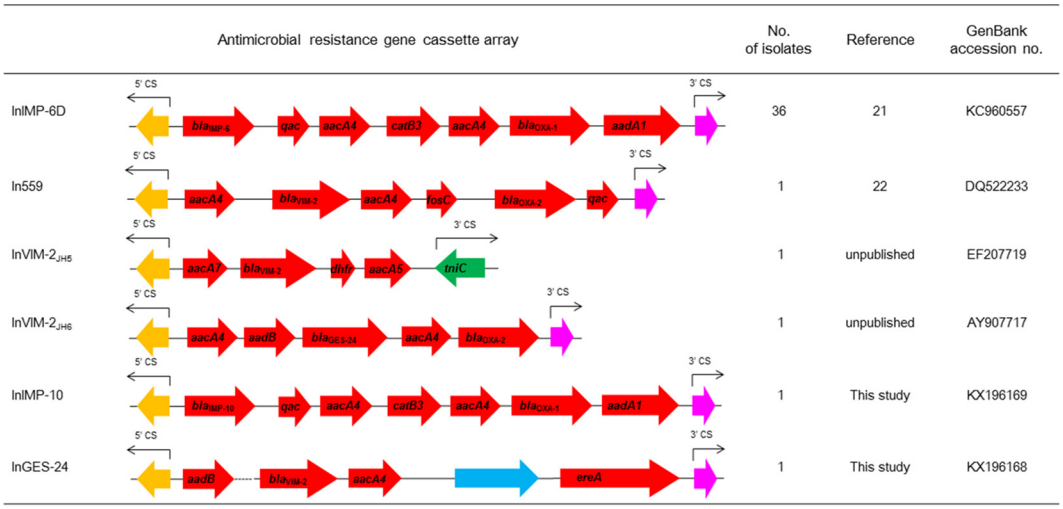


FIG 3 Schematic representation of class 1 integrons carrying the carbapenemase gene cassettes. Yellow arrows, integrase; red arrows, genes for resistance; magenta arrows, *qacEΔ1*; green arrow, *tniC* gene; blue arrow, a gene for tyrosine recombinase XerD; dotted line, unknown nucleotide sequences.

the $tRNA^{Gly}$ gene, but the DR was 27 bp, AGGGTTCGATTCCC TTCGCCCGCTCCA (Fig. 4A). The G+C content of the PAGI-16 was 61.3%, slightly lower than that of the PAGI-15. This genomic island shared the backbone with PAGI-16, presenting 99.99% nucleic acid identity excluding the class 1 integron and a short putative prophage possessing nine ORFs located in the 3′ end of the genomic island (Fig. 4A). PAGI-16 contained 116 putative ORFs, mostly shared with PAGI-15 except 11 antimicrobial resistance genes, two copies of *aacA4*, *aadB*, *strA*, *strB*, *tet*(*G*), *cmx*, two copies of *sul1*, bla_{OXA-2}, and bla_{GES-24}.

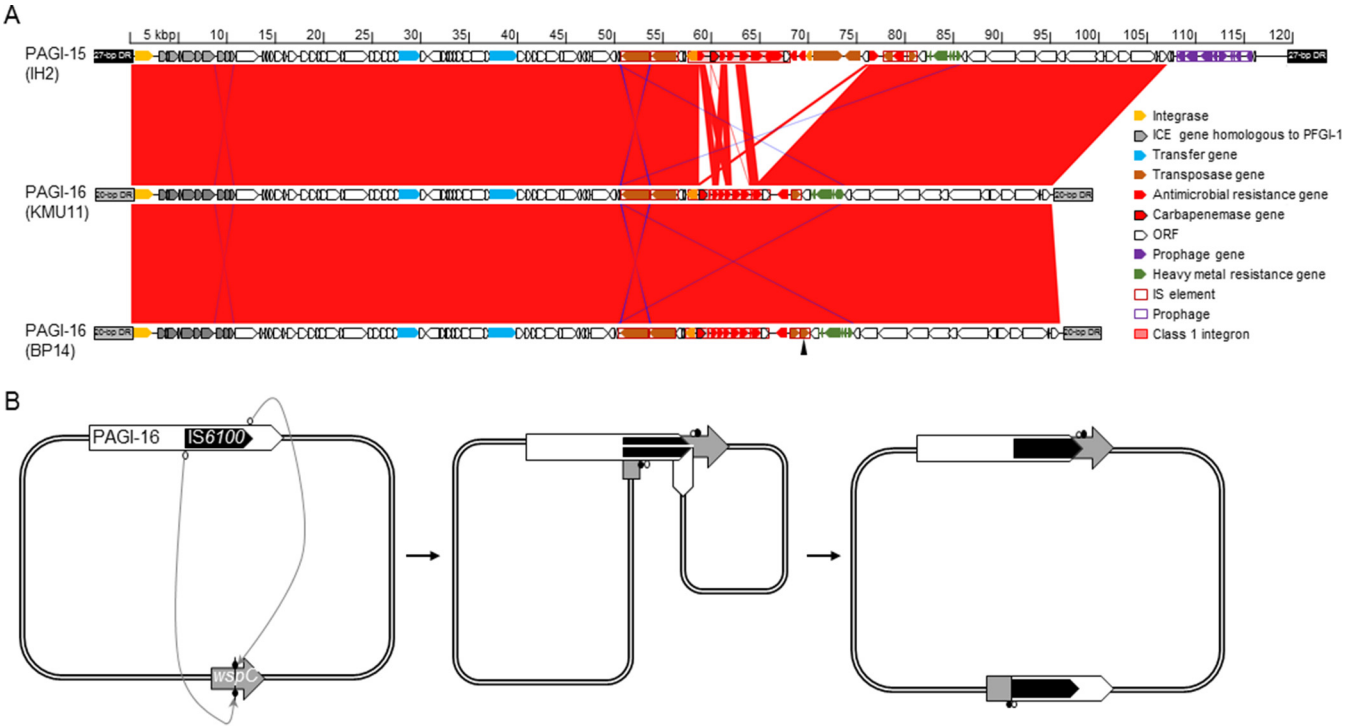


FIG 4 Schematic representation of genomic islands in strains IH2, KMU11, and BP14. (A) The sequences of genomic islands of *P. aeruginosa* isolates were aligned using BLASTN and compared using Artemis Comparison Tool (32). The conserved regions (>95% identity) are indicated in red. Arrowhead, location of chromosomal inversion due to duplication-insertion of IS*6100*. (B) Schematic mechanism for the large chromosomal inversion mediated by duplication of IS*6100*. In a chromosome carrying PAGI-16, catalyzed 3′-OH groups of IS*6100* (white circle) targeted the 5′ phosphate in the *wspC* gene (black circle) located elsewhere. The replication broke the cointegration, leading to the duplication in the opposite direction of IS*6100* and a chromosomal inversion. The final chromosomal structure became 5′PAGI-16-IS*6100*-5′Δ*wspC* and 3′PAGI-16-IS*6100* (opposite direction)-3′Δ*wspC*. White arrow, PAGI-16; black arrow, IS*6100*; gray arrow, *wspC*.

DISCUSSION

A nationwide survey in 2009 reported that the carbapenem resistance rate in *P. aeruginosa* was 35.8% (138/386) and 8.0% (31/386) of the strains produced carbapenemases IMP-6 ($n = 30$) and VIM-2 ($n = 1$) (8). In this surveillance, a similar rate of CP-PA was found, 9.5% (41/431). Among CP-PA strains, prevalence of bla_{IMP-6}-producing *P. aeruginosa* has been detected exclusively in South Korea. IMP-6 production by the bacterial host results in a higher MIC for meropenem than for imipenem. This is due to the Ser216-Gly substitution, which results in increased hydrolysis of meropenem compared with that of imipenem (26). Interestingly, meropenem is highly recommended for treating Gram-negative bacterial infections, rather than imipenem (27), and meropenem usage is increasing faster than that of imipenem (28). It is likely that antibiotic selective pressure leads to the predominance of IMP-6 in *P. aeruginosa*.

Clonal diversity of the epidemic isolates, which consisted mostly of ST235 isolates presenting indistinguishable PFGE patterns, allowed us to understand that the dissemination of IMP-6 was mostly by clonal dissemination of the *P. aeruginosa* ST235 isolates possessing the gene. The gene cassette array added more evidence for that, showing all IMP-6-positive strains possessed an identical class 1 integron, InIMP-6D (*intI1-*bla_{IMP-6}*-qac-aacA4-catB3-aacA4-*bla_{OXA-1}*-aadA1*). Interestingly, the gene cassette array of this class 1 integron resembled that of the integron *intI1-*bla_{IMP-6}*-qac-aacA4-*bla_{OXA-1}*-aadA1* in epidemic strains of *P. aeruginosa* isolated in 2009 (8) (Fig. 3).

The second most prevalent carbapenemase was VIM-2, although it was identified in only three isolates. The bla_{VIM-2} gene was sporadically identified to be associated with unrelated integrons in *P. aeruginosa* of two different STs, ST235 and ST244, each with different PFGE patterns (Fig. 2). These results suggest that bla_{VIM-2} dissemination was due not to clonal spread but rather to an occasional occurrence. Interestingly, In559, one of the three class 1 integrons carrying the bla_{VIM-2} gene cassette, had a distinct 3′ end, matured by *tniC* of Tn*5090* instead of the usual 3′ CS with *qacEΔ1*. This finding likely indicates that In559 has been developed through a different evolutionary scheme used by Tn*5090*. A similar bla_{VIM-2} gene cassette-carrying integron, which was identified as an ancestral class 1 integron 3′ ended by the *tniC* gene, was identified in a *P. aeruginosa* strain isolated in India in 2003 (23). The precise epidemiological relationship of the unusual integrons in our strain and in the Indian strain is unclear, since no ST information could be obtained for the case.

This surveillance study enabled the first observation of IMP-10 and GES-24, indicating diversification of carbapenemases in *P. aeruginosa* in South Korea. The bla_{IMP-10}-associated integron has previously been identified in *P. aeruginosa* in Japan (29). We initially suspected that the first identification of bla_{IMP-10} reflected a traveler carrier; however, after inspecting the genomic environment of this gene, we concluded that this gene appearance was a result of spontaneous nucleotide substitutions independently from bla_{IMP-1}, a G640A substitution resulting in Gly216-Ser mutation for IMP-6 in PAGI-16 of KMU11 and G145T leading to Val49-Phe mutation in IMP-10 in PAGI-16 of BP14. Indeed, this conclusion is supported by two additional pieces of evidence. First, InIMP-6D and InIMP-10 have the same genetic context, with the exception of the MBL gene cassettes (bla_{IMP-6} and bla_{IMP-10}) between the 5′ CS and the 3′ CS (Fig. 3). Second, PAGI-16, which was found to carry the bla_{IMP-6} gene (GenBank accession number KX196167), and PAGI-16, which was found to carry the bla_{IMP-10} gene (GenBank accession number KX196169), exhibited a high degree of homology based on WGS results and shared most of the conserved regions (95% identity) (Fig. 4A).

So far, six GES β-lactamases, namely, GES-2, -4, -5, -6, -14, and -18, have been shown to present detectable carbapenemase activity (6). This activity is predominantly a factor of substitutions of amino acid residues 104 and 170. The GES-24 enzyme has Gly170, like GES-5, which results in high affinity for carbapenems and high turnover rates due to the low rate constants for acylation and deacylation (30). The GES-5 producers *Klebsiella pneumoniae* and *E. coli* have been found in South Korea (31), but the genes were on very different integrons. The genes for GES-24 in *Enterobacter cloacae* and *Acinetobacter baumannii* from Japan are available in GenBank (last updated on 13 May 2016), but to the best of our knowledge, this is the first description of GES-24 in *P. aeruginosa*.

Interestingly, PAGI-15 and PAGI-16 were found to differ only by their integrons. It is likely that the two genomic islands derived from a common origin. They once possessed a backbone possessing a *clc*-like element, into which two unrelated integrons were later integrated. Moreover, PAGI-16 in one isolate was found to have split into two parts by large chromosomal inversion that resulted in duplication-insertion of the IS*6100* gene in the opposite direction (Fig. 4B). Similar IS*6100*-mediated large chromosomal inversions have been found in *P. aeruginosa* clinical isolates and are likely to be involved in phenotypic adaptations of the different strains to the environment (25).

PAGI-15 and -16 in this study were found in *P. aeruginosa* belonging to ST244 and ST235. These genomic islands have shared origin evidenced by the same backbone in spite of the distinct STs of the host *P. aeruginosa*. Interestingly, a genomic island having a same backbone is found also in the chromosome of *P. aeruginosa* ST395 isolated in France in 1997 (GenBank accession number CP013993). The genomic island has a class 1 integron possessing one gene cassette, *aadB*. This highly mobile genomic island is likely to have a key role for capturing and disseminating the multiple antibiotic resistance genes in *P. aeruginosa* belonging to various STs, similar to the case with PAGI-1 and -2 (13).

In conclusion, our data indicate that IMP-6 is highly prevalent in CP-PA ST235 isolates. Moreover, our results clearly demonstrate that WGS methodology can be used to identify the genomic environments associated with resistance determinants, which promises to shed light on many epidemiological questions regarding the mechanism of dissemination of resistance determinants.

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