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Persistence and Epidemic Propagation of a *Pseudomonas aeruginosa* Sequence Type 235 Clone Harboring an IS*26* Composite Transposon Carrying the bla_{IMP-1} Integron in Hiroshima, Japan, 2005 to 2012

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A 9-year surveillance for multidrug-resistant (MDR) *Pseudomonas aeruginosa* in the Hiroshima region showed that the number of isolates harboring the metallo-β-lactamase gene bla_{IMP-1} abruptly increased after 2004, recorded the highest peak in 2006, and showed a tendency to decline afterwards, indicating a history of an epidemic. PCR mapping of the variable regions of the integrons showed that this epidemic was caused by the clonal persistence and propagation of an MDR *P. aeruginosa* strain harboring the bla_{IMP-1} gene and an aminoglycoside 6′-*N*-acetyltransferase gene, *aac(6′)-Iae* in a class I integron (In113), whose integrase gene *intI1* was disrupted by an IS*26* insertion. Sequence analysis of the representative strain PA058447 resistance element containing the In113-derived gene cassette array showed that the element forms an IS*26* transposon embedded in the chromosome. It has a Tn*21* backbone and is composed of two segments sandwiched by three IS*26*s. In Japan, clonal nationwide expansion of an MDR *P. aeruginosa* NCGM2.S1 harboring chromosomally encoded In113 with intact *intI1* is reported. Multilocus sequence typing and genomic comparison strongly suggest that PA058447 and NCGM2.S1 belong to the same clonal lineage. Moreover, the structures of the resistance element in the two strains are very similar, but the sites of insertion into the chromosome are different. Based on tagging information of the IS*26* present in both resistance elements, we suggest that the MDR *P. aeruginosa* clone causing the epidemic in Hiroshima for the past 9 years originated from a common ancestor genome of PA058447 and NCGM2.S1 through an IS*26* insertion into *intI1* of In113 and through IS*26*-mediated genomic rearrangements.

Pseudomonas aeruginosa is an opportunistic human pathogen and a leading cause of nosocomial infections. It also causes mastitis in dairy cows as mild chronic persistent inflammation (1). Carbapenems have been used to treat *P. aeruginosa* infections; however, *P. aeruginosa* isolates resistant to carbapenems are reported in many countries and an acquired resistance to carbapenems by metallo-β-lactamase (MBL) genes causes a serious therapeutic problem in clinical settings. Carbapenem-resistant *P. aeruginosa* strains producing MBLs were first discovered in Japan (2, 3) and have since been reported in many countries all over the world (4).

Specifically, IMP, VIM, SPM, and NDM are the most important types of MBL with worldwide distribution, and their genes are frequently carried on class 1 integrons (4, 5). Class 1 integrons are often found on plasmids or as a part of transposons or resistance genetic elements that are able to integrate into chromosomal elements, resulting in an increase in the number of resistant Gram-negative bacilli (6). MBL-producing *P. aeruginosa* strains often behave as multidrug-resistant (MDR) strains. The recent increase of nosocomial infections caused by MDR *P. aeruginosa* has raised a serious concern.

In the Hiroshima prefecture, we have conducted a multihospital surveillance for drug-resistant *P. aeruginosa* in nine hospitals and reported an increase of MDR *P. aeruginosa* carrying bla_{IMP-1} from 2004 to 2006 (7). According to PCR mapping of the variable regions of the integrons, we classified the isolates into six bla_{IMP-1} integron cassette types and designated the isolate types A through F. We reported an increase of type F MDR *P. aeruginosa* harboring the bla_{IMP-1} and a unique aminoglycoside N-acetyltransferase gene, *aac(6′)-Iae*, in the class I integron named In113, whose integrase gene *intI1* was disrupted by IS*26* insertion. In113 was first discovered in isolate NCGM2.S1 (previously named IMCJ2.S1) (8) from a nosocomial *P. aeruginosa* outbreak occurring in the neurosurgery ward of a hospital in Sendai, Miyagi prefecture, 600

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km from Hiroshima, in 2002 (9). Further epidemiology studies by the same research group demonstrated that MDR *P. aeruginosa* carrying *aac(6′)-Iae* with a pulse type identical to that of NCGM2.S1 was prevalent in Miyagi (10) and Tokyo (11), suggesting that MDR *P. aeruginosa* NCGM2.S1 was widely disseminated in Japan. Our initial surveillance from 2004 to 2006 also identified MDR *P. aeruginosa* harboring In113 with an intact *intI1* gene, classified as type E (7).

Here, we describe further longitudinal molecular epidemiology studies of drug-resistant *P. aeruginosa* in the Hiroshima region and demonstrate an epidemic by a single clonal type F MDR *P. aeruginosa* that has continued for 9 years. Sequence studies of the type F representative strain PA058447 resistance element carrying bla_{IMP-1} showed that the element forms an IS*26* transposon with a Tn*21* backbone. Our data suggest that type F MDR *P. aeruginosa*, prevalent in the Hiroshima region, and strain NCGM2.S1, carrying In113 and prevalent in the Tohoku region, share the same clonal origin.

MATERIALS AND METHODS

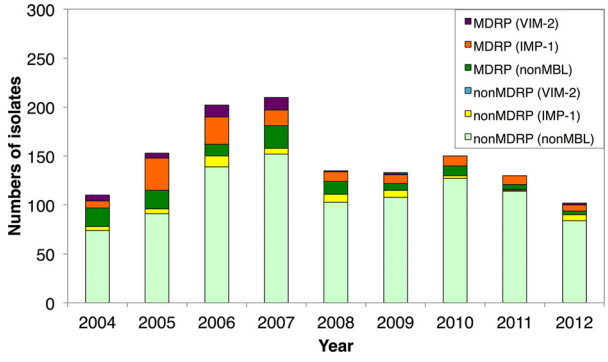
Collection of *P. aeruginosa* isolates and antimicrobial susceptibility testing. Nonrepetitive *P. aeruginosa* isolates were obtained from patients from eight major hospitals in Hiroshima during July 2004 to December 2012. Identification of *P. aeruginosa* was performed by the submitting hospital laboratories. They were cultured on nalidixic acid-cetrimide (NAC) agar (Eiken, Tokyo, Japan) and verified using PCR amplification with primers specific for *P. aeruginosa* 16S rRNA. Imipenem- or ceftazidime-resistant *P. aeruginosa* isolates were selected when the MIC of imipenem was ≥8 mg/liter or that of ceftazidime was ≥16 mg/liter. MICs were determined using the broth microdilution method from the Clinical and Laboratory Standards Institute (CLSI) (12). The antibiotics tested were ciprofloxacin (Meiji Seika Pharma, Ltd., Tokyo), imipenem (Banyu Pharmaceutical Co., Ltd., Tokyo), and amikacin (Banyu Pharmaceutical Co., Ltd., Tokyo).

Criteria for MDR *P. aeruginosa* were in accordance with the Law Concerning the Prevention of Infections and Medical Care for Patients with Infections from the Japanese Ministry of Health, Labor, and Welfare, whereby the criteria are resistance to imipenem (MIC ≥ 16 mg/liter), amikacin (MIC ≥ 32 mg/liter), and ciprofloxacin (MIC ≥ 4 mg/liter). The criterion for amikacin resistance (MIC ≥ 64 mg/liter) was different from that given in the CLSI guidelines (MIC ≥ 32 mg/liter) (more stringent).

MBL screening test with an inhibitor using a double-disc synergy test with two Kirby-Bauer discs. All of the imipenem- or ceftazidime-resistant *P. aeruginosa* isolates were screened for the presence of MBL using sodium mercaptoacetic acid (SMA) as previously described (13).

PCR detection and characterization of the variable regions of the bla_{IMP-1}-containing integrons. The primers used were described previously (7). PCR amplification was performed using TaKaRa Ex *Taq* DNA polymerase (TaKaRa, Tokyo, Japan) with 25 cycles as follows: denaturing at 98°C for 10 s, annealing at 50°C for 30 s, and polymerization at 72°C for 1 min. The structure of the variable regions of the bla_{IMP-1}-containing integrons was determined using a PCR mapping approach with primers designed from the conserved integron sequences flanking the cassette array as previously described (7).

Fosmid library and screening. A fosmid library of *P. aeruginosa* PA058447 was constructed using a CopyControl fosmid library production kit (Epicentre) according to the manufacturer's instructions. The genomic DNA was sheared using a 26-gauge syringe. After blunting and phosphorylation of the DNA fragments, the fragments were separated by pulsed-field gel electrophoresis with 1% certified low-melt agarose (Bio-Rad Laboratories, Inc., Tokyo, Japan). Fragments of ~40 kb were recovered from the gel using GELase (Epicentre). After the DNA solution was concentrated with a Microcon YM-100 filter (Millipore), the fragments were ligated to the pCC1FOS vector, packaged into phage particles, and transfected into *Escherichia coli* EPI300-T1 cells. We collected 2,880 chloramphenicol-resistant clones. Fosmid clones containing bla_{IMP-1} were further selected on LB agar plates containing ampicillin (30 mg/liter).



	2004	2005	2006	2007	2008	2009	2010	2011	2012
MDRP/all (%)	29.1	37.3	25.7	23.8	17.7	13.5	13.3	10.8	11.8
MBLs/all (%)	15.5	28.1	25.3	15.1	14.1	13.5	8.7	8.5	13.7
MBL/MDRP (%)	40.6	66.7	76.9	53.1	45.8	61.1	50	64.3	66.7

FIG 1 Annual number of imipenem- or ceftazidime-resistant *P. aeruginosa* isolates. The annual number of MDR *P. aeruginosa* isolates (MDRP) or metallo-β-lactamase-producing isolates (MBL) with imipenem- or ceftazidime-resistant *P. aeruginosa* and the annual number of MBL in MDRP are shown.

Nucleotide sequencing, assembly, and genome comparison. The nucleotide sequence of the cloned fragment into the fosmid vector was determined using the random shotgun sequencing method described previously (14). Collected sequences were assembled using the Sequencher DNA sequencing software (v3.0; Gene Codes). Gaps were closed by direct sequencing of the PCR products amplified with oligonucleotide primers designed to anneal to each end of the neighboring contigs. Initially, potential protein-encoding regions (open reading frames [ORFs]) that were ≥150 bp long were identified using MetaGeneAnnotator (15) and the In Silico Molecular Cloning software package, Genomics Edition (InSilico Biology Inc., Yokohama, Japan). Each ORF was reviewed manually for the presence of a ribosomal binding sequence. Functional annotation was assigned based on homology searches against the GenBank nonredundant protein sequence database using the program BLASTP (16). Protein and nucleotide sequences were compared with those in the sequence databases using the BLAST and FASTA programs at the DDBJ (DNA Data Bank of Japan; http://www.ddbj.nig.ac.jp/). The draft genome sequence of PA058447 was determined using Illumina MiSeq (Nextera paired-end library; 3,525,294-bp sequences; 66.49-fold coverage) sequencing platform. The standard protocol with the Nextera XT DNA sample preparation kit was used. Whole-genome comparison of PA058447 and NCGM2.S1 was performed with BRIG (17) using unassembled Illumina reads of PA058447 and the genome sequence of NCGM2.S1 mapping with reference to the genome of PAO1.

Nucleotide sequence accession number. The nucleotide sequence described here has been deposited in GenBank under accession number AB983593.

RESULTS AND DISCUSSION

Screening for drug-resistant strains. A total of 1,434 *P. aeruginosa* isolates resistant to imipenem or ceftazidime found during July 2004 to December 2012 were from nine hospitals (identified by the letters "a" to "i") in Hiroshima. The strains were isolated most commonly from either sputum or urine samples (619 [43.2%] or 345 [24.1%], respectively). The annual numbers of imipenem- or ceftazidime-resistant *P. aeruginosa* strains continuously increased during the first 4 years of the study, from 110 in

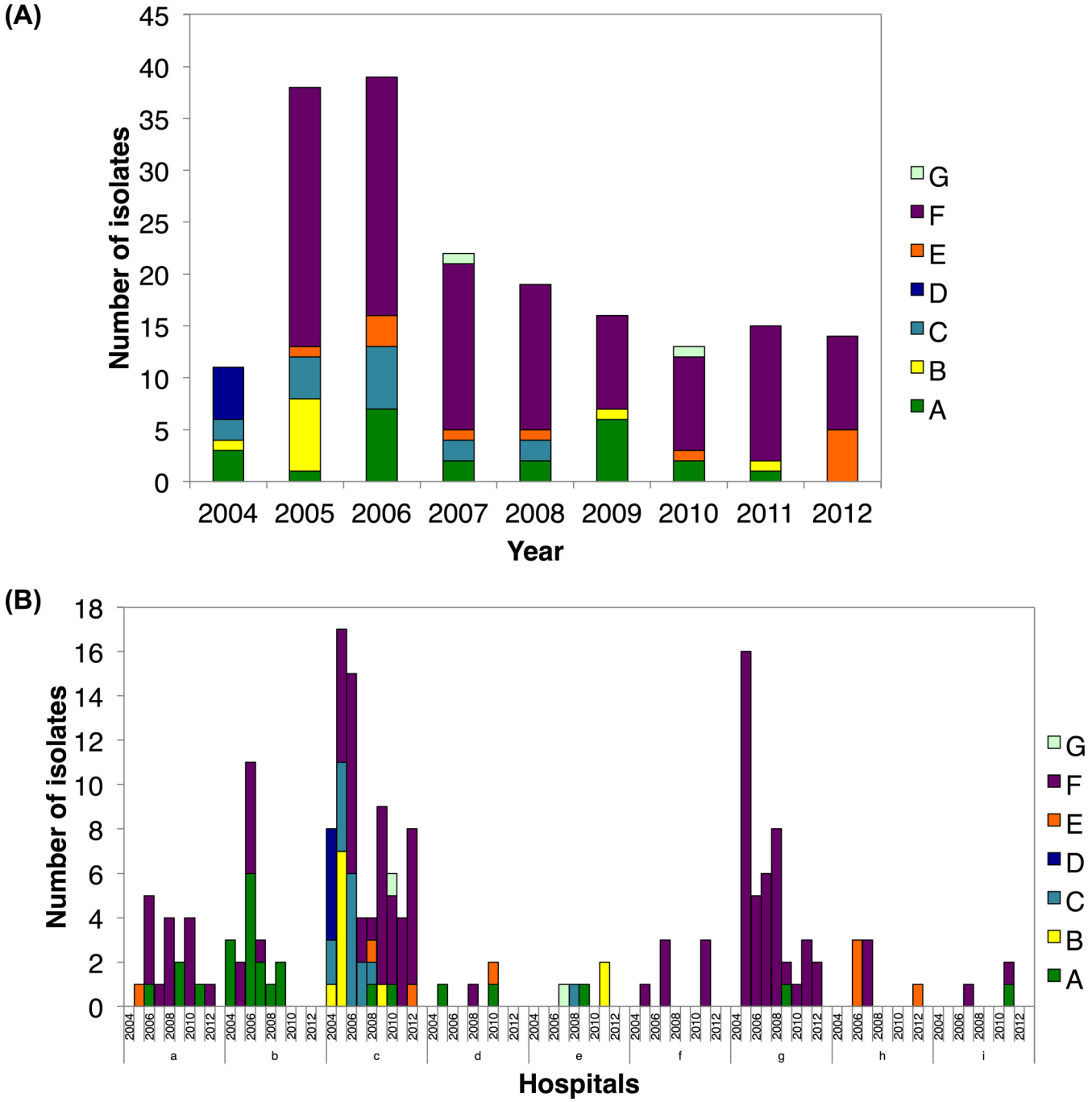


FIG 2 (A) Annual survey of the number and the integron type of *P. aeruginosa* isolates carrying bla_{IMP-1} integron. (B) Annual survey of isolation incidence of *P. aeruginosa* carrying the bla_{IMP-1} integron in each hospital (a to i) participating in this study. Letters A to H indicate the integron types shown in Fig. S1 in the supplemental material.

2004 to 206 in 2007, and then fluctuated from 2008 to 2012 (Fig. 1). The prevalence of MDR *P. aeruginosa* in imipenem- or ceftazidime-resistant isolates continued at a higher ratio of over 20% in the first 4 years, with a maximum of 37.3% in 2005. After 2008, the rate started to gradually decline and reached 12% in 2012, but further follow-up may be necessary to infer the prevalence of MDR *P. aeruginosa* in this region. Similarly the prevalence of MBL producers among the imipenem- or ceftazidime-resistant isolates reached a maximum of 28.1% in 2005 and gradually decreased and was 13.2% in 2012. The prevalence of MBL producers among MDR *P. aeruginosa* strains dramatically increased during the first 3 years, from 40.6 to 76.9%, but this high rate did not recede thereafter and remained at 50 to 60%, even though the prevalence of MBL in imipenem- or ceftazidime-resistant isolates significantly decreased. Our previous studies demonstrated that all of the suggested MBL-producing strains were positive for either

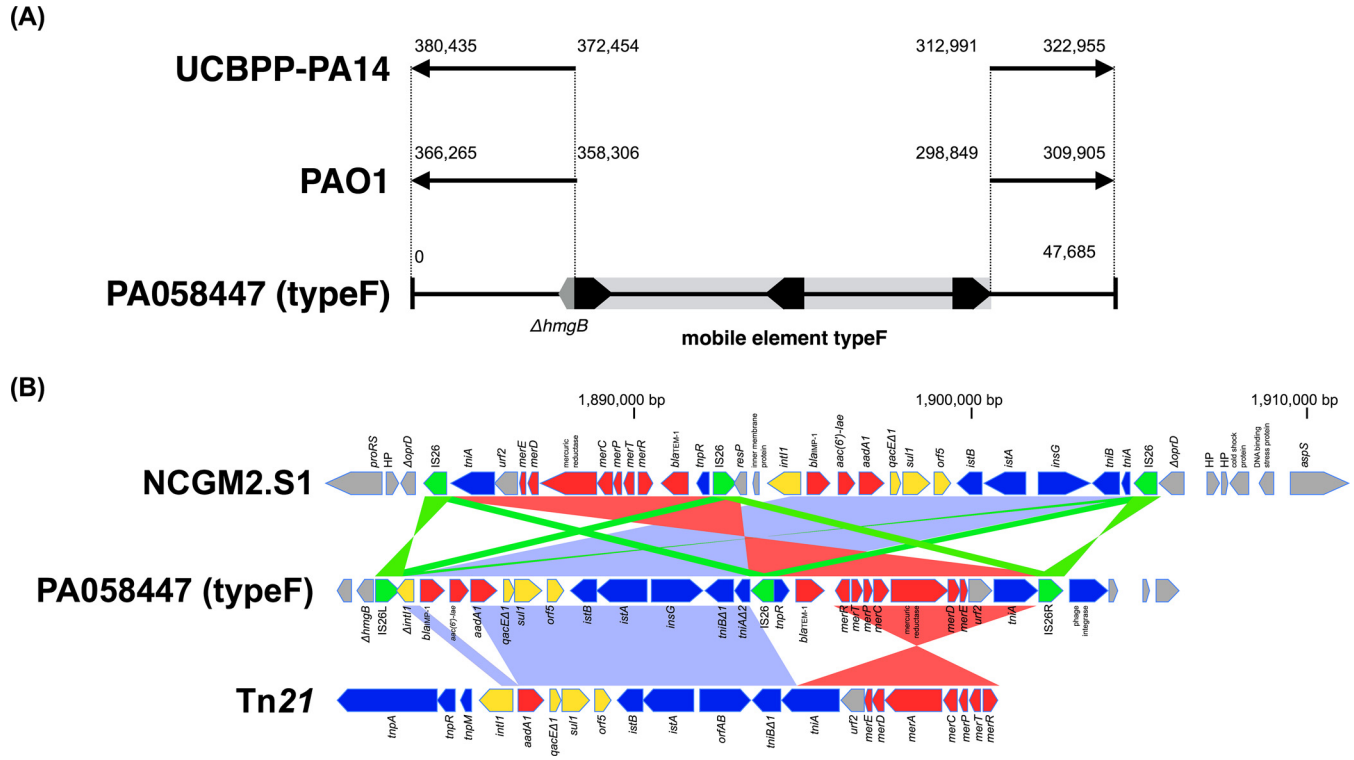


FIG 3 (A) Schematic representation of genomic rearrangement due to mobilization of the type F resistance element. Black pentagons indicate IS*26*s and their orientations. *hmgB*, fumarylacetoacetate hydrolase gene. Alignments of the complete nucleotide sequence of the cloned fragment from PA058447 and genome sequences of PAO1 and UCBPP-PA14 are shown. The gray bar indicates the type F resistance element. (B) Structural comparison of PA058447 type F resistance element (IS*26* transposon), NCGM2.S1 resistance element (IS*26* transposon), and Tn*21*. Color shading indicates homologous regions. Coding sequences are shown as pentagons (green, IS*26*; blue, transposon; yellow, integron; red, antibiotic resistance gene; gray, other function or hypothetical protein).

bla_{IMP-1} or bla_{VIM-2} (7). Among them, bla_{IMP-1}-positive strains have been isolated from various hospitals. Follow-up studies indicated that this tendency continued to 2012. We detected six integron polymorphs in bla_{IMP-1} integron (types A to F) during 2004 to 2006, and another type (denoted as type G) was added in 2012 (18) (Fig. 2). Cumulative data of annual isolation of bla_{IMP-1}-positive strains indicated that they reached a maximum in 2006 and gradually decreased to the level of ca. 15 strains per year, which is still higher than that in 2004 (Fig. 2A). After 2005, type F has been overrepresented in bla_{IMP-1}-positive strains. Type F was first isolated in four of eight hospitals in 2005, but the number of hospitals with a history of isolation of this type reached seven within the 9-year period (Fig. 2B).

Resistance element type F. The type F isolates shared identical bla_{IMP-1} integron gene cassettes and further downstream sequences with the type E, except that the 5′-*intI1* gene is truncated by an IS*26* insertion in type F (see Fig. S1 in the supplemental material). The insertion of IS*26* into *intI1* resulted in the formation of a composite transposon in which two IS*26*s are bracketing the variable region of In113 and flanking genes derived from In2 (see Fig. S1 in the supplemental material) (19). To further delineate the overall structure of the type F resistance element containing bla_{IMP-1}, a fosmid library for strain PA058447 was constructed. From 2,880 fosmid clones, 26 clones including the bla_{IMP-1} gene were selected on LB agar plates containing ampicillin. Complete sequencing of one of the cloned 47.7-kb fragments showed that the overall structure of the resistance element is composed of two segments sandwiched by three IS*26* elements embedded in the chromosome disrupting the gene fumarylacetoacetate hydrolase (*hmgB*) (Fig. 3A and B). Flanking sequences of the resistance element clearly demonstrated that the element's 3′ downstream region had an orientation opposite to that of PAO1 and UCBPP-PA14 sequences, suggesting that there was a process of genomic inversion during the acquisition of the type F resistance element (Fig. 3A). The left segment (3′) of the resistance element corresponds to the genetic content of the type F integron (7) (Fig. 3B). The right segment (5′) has 11 ORFs, most of which constitute the *mer* operon of Tn*21* (19) (Table 1). A homology search of nucleotide sequences of the overall resistance element indicated that its backbone is part of Tn*21*, especially In2 and the *mer* gene operon segmented by IS*26*s (Fig. 3B). Previously, the mobile element of *P. aeruginosa* carrying bla_{IMP-1} (strain 101/1477) isolated in Japan has been characterized (20). It is carried on a plasmid and located on an integron, In31. In2 and In31 share identical sets of 25-bp inverted repeats (IRi and IRt) sandwiching integrons, but the nucleotide sequence flanking IRt of In31 is different from that of In2 and that found in the type F right segment, suggesting that the type F element and In31 are evolutionary distinct.

Class I integron plays an important role in the dissemination of antimicrobial-resistance genes and horizontal gene transfer. The recent emergence and prevalence of drug-resistant *Acinetobacter baumannii* carrying class I integron with a gene array identical to that of *P. aeruginosa* suggests the efficient transfer of class I integrons among *P. aeruginosa* and *A. baumannii*. So far, In113 has

TABLE 1 Features of ORFs around type F integron (GenBank accession no. AB983593)

ORF	Location (bp)	Strand	Gene	Size (bp)	Length (aa)[a]	Translation signal sequence[b]	Homologue as determined by BLAST and/or FASTA: Source	Description(s)	Identity (%)	Overlap[c] (aa)	Accession no.
1	595–1121	−	Δ*hmgB*	527			*P. aeruginosa* (PAO1)	Fumarylacetoacetate hydrolase (5′-side 139 bp truncated)	99	174/221	AE004091
2	1185–1889	+	Transposase gene	705	234	GGAGCTGCAC**ATG**	*P. aeruginosa*	Insertion sequence 26 transposase	100	234/234	AB104852
3	1942–2316	−	Δ*intI1*	375			*P. aeruginosa*	Integrase1 (3′-side 639 bp truncated)	100	125/337	AF313472
4	2469–3209	+	bla_{IMP-1}	741	246	AAGGAAAAGT**ATG**	*P. aeruginosa*	Metallo-β-lactamase bla_{IMP-1}	100	246/246	AB104852
5	3363–3914	+	*aac6′-Iae*	552	183	AAGAGGTTTT**ATG**	*P. aeruginosa*	Aminoglycoside acetyltransferase 6′-Iae (*aac6′-Iae*)	100	183/183	AB104852
6	3989–4780	+	*aadA1*	792	263	TAAACATC**ATG**	*P. aeruginosa*	Aminoglycoside adenyltransferase A1 (*aadA1*)	100	263/263	AB104852
7	4944–5291	+	*qacEΔ1*	348	115	GGAGATATATC**ATG**	*P. aeruginosa*	Quaternary ammonium compound reistance (*qacEΔ1*)	100	115/115	AB104852
8	5285–6124	+	*sul1*	840	279	GGAGGCCGACGCC**ATG**	*P. aeruginosa*	Sulfonamide resistance protein (*sul1*)	100	279/279	AB104852
9	6252–6752	+	*orf5*	501	166	AGGGGAGCGA**ATG**	*P. aeruginosa*	Acetyltransferase (GNAT) family protein	100	166/166	AB104852
10	6928–7710	−	*istB*	783	260	AAGGAGAGCCCATG**ATG**	*P. aeruginosa*	IstB-like ATP binding family protein	100	260/260	AB104852
11	7700–9223	−	*istA*	1,524	507	GGAGAAATCAAGGA**GTG**	*P. aeruginosa*	Possible transposase of IS*1326*	100	507/507	AB104852
12	9346–10890	+	*insG*	1,545	514	AGGAGTAGTTC**ATG**	*P. aeruginosa*	Transposase for IS*1353*	100	514/514	CP008739
13	10941–11801	−	*tniB*	861	286	GAGGAGTGGTAGCC**GTG**	*P. aeruginosa*	Transposition protein TniB	100	286/286	AB104852
14	11804–12231	−	Δ*tniA*	428			*K. pneumoniae*	Transposition protein TniA-C-terminal part			KF976462
15	12292–12996	−	Transposase gene	705	234	GGAGCTGCAC**ATG**	*P. aeruginosa*	Insertion sequence 26 transposase	100	234/234	AB104852
16	13060–13452	+	*tnpRΔ2*	393			*Salmonella enterica*	Resolvase *tnpRΔ2*			EU219534
17	13635–14495	+	bla_{TEM-1}	861	286	AAGGAAGAGT**ATG**	*P. aeruginosa*	Extended-spectrum β-lactamase bla_{TEM-1}	100	286/286	X54607
18	14777–15211	−	*merR*	435	144	GGAGTCAAGCGAT**ATG**	*Salmonella enterica*	Activator/repressor of *mer* operon (*merR*)	100	144/144	AM991977
19	15283–15633	+	*merT*	351	116	AAGGGCCAACGT**ATG**	*Salmonella enterica*	Mercuric ion transport protein (*merT*)	100	116/116	AM991977
20	15647–15922	+	*merP*	276	91	TGGATTTCCCT**ATG**	*Salmonella enterica*	Periplasmic mercuric ion binding protein (*merP*)	100	91/91	AM991977
21	15958–16380	+	*merC*	423	140	GAGAGCCGCTTC**ATG**	*Salmonella enterica*	Transmembrane protein (*merC*)	100	140/140	AM991977
22	16432–18126	+	*merA*	1,695	564	AAGGAACGATGGT**ATG**	*Salmonella enterica*	Mercuric ion reductase (*merA*)	100	564/564	AM991977
23	18144–18506	+	*merD*	363	120	AAGGAGGTGTGCG**ATG**	*Salmonella enterica*	Putative secondary regulatory protein (*merD*)	100	120/120	AM991977
24	18503–18739	+	*merE*	237	78	AGGAGGCATTGCC**GTG**	*Salmonella enterica*	Mercuric resistance protein (*merE*)	100	78/78	AM991977
25	18736–19443	+	*urf2*	708	235		*Salmonella enterica*	EAL domain protein Urf2 (*urf2*)	100	235/235	AM991977
26	19482–20786	+	*tniAΔ1*	1,305	434	GAGGTGAGC**ATG**	*Salmonella enterica*	Transposition protein TniAΔ1	100	434/434	EU219534
27	20833–21537	+	Transposase gene	705	234	GGAGCTGCAC**ATG**	*P. aeruginosa*	Insertion sequence 26	100	234/234	AB104852
28	21733–22893	+	Phage integrase gene	1,161	386	GAGGCATTTGCC**ATG**	*P. aeruginosa*	Phage integrase	97	386/387	EU595745

[a] aa, amino acids.
[b] Underlining indicates ribosome binding site; boldface indicates start codon.
[c] Overlap indicates the number of overlapping amino acids/total number of amino acids.

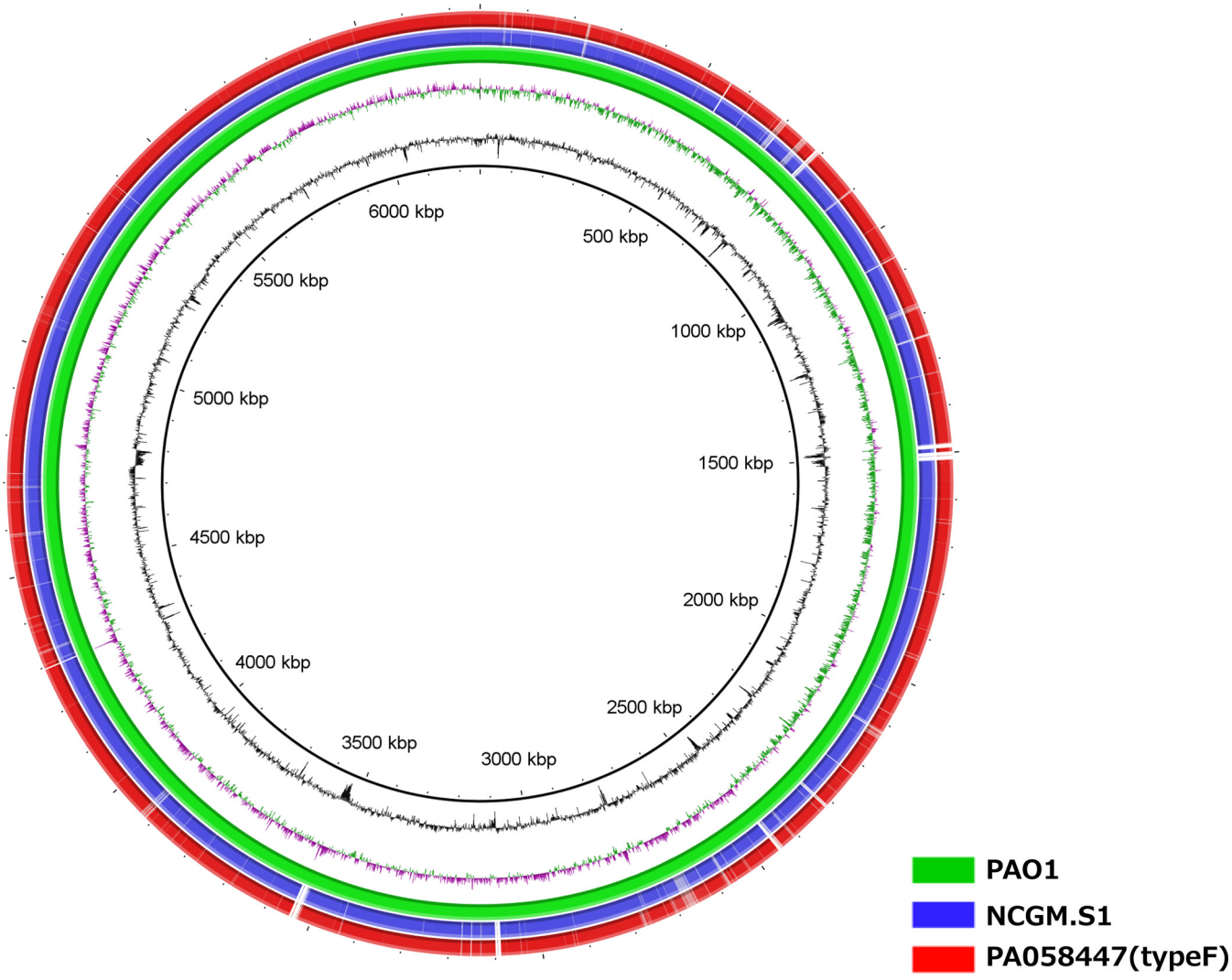


FIG 4 Comparison of genome sequences of PA058447 and NCGM2.S1. Unassembled sequences of *P. aeruginosa* PA058447 or *P. aeruginosa* NCGM2.S1 genome sequences were mapped and compared to the complete genome sequence of *P. aeruginosa* PAO1. After assembly, contigs were ordered and compared to the complete *P. aeruginosa* PAO1 genome. The innermost rings represent GC skew (purple/green) and GC content (black).

not been identified in *A. baumannii*, but the prevalence of MDR *P. aeruginosa* carrying In113 in Japan may pose a threat, indicating a potential for the emergence of *A. baumannii* carrying In113 in future.

Comparison of PA058447 type F resistance element and NCGM2.S1 In113 resistance element. We compared the overall structure of the type F resistance element to the NCGM2.S1 In113 resistance element using the genome sequence of NCGM2.S1 (8). Both elements showed high similarity in gene organization: the In113 resistance element is also composed of two segments sandwiched by three IS*26* elements and embedded in the chromosome (Fig. 3B). The left segment of the In113 resistance element has inversion symmetry to the right segment of the type F resistance element. Conversely, the right segment of the NCGM2.S1 resistance element contains the left segment of type F resistance element and a few additional 5′ flanking genes. The In113 resistance element of NCGM2.S1 has three copies of IS*26*. Like those of the type F resistance element, two genes at both ends are in direct relative orientation, flanking two interstitial segments forming a composite transposon of the IS*6* family (21). This transposon is inserted into *oprD*, resulting in complete disruption of the gene. Conversely, PCR analysis revealed that *oprD* in strain PA058447 carrying the type F resistance element was intact. Therefore, the type F resistance element is not a direct sibling of the In113 resistance element by simple insertion of additional IS*26* into the *intI1* gene of In113.

Hypothesis for the molecular evolution of type F. Multilocus sequence typing (MLST) indicated that MDR *P. aeruginosa* type E (carrying In113) and type F, isolated in Hiroshima, and those strains carrying the In113 resistance element, isolated in the Tohoku area, belonged to the same ST, ST235. To further compare the chromosomal backgrounds of type F and MDR *P. aeruginosa* carrying the In113 resistance element isolated in Tohoku, we obtained a draft sequence of strain type F (PA058447) using a new-generation sequencer. Figure 4 shows BLASTn comparisons between the draft genome sequence of strain PA058447 and the

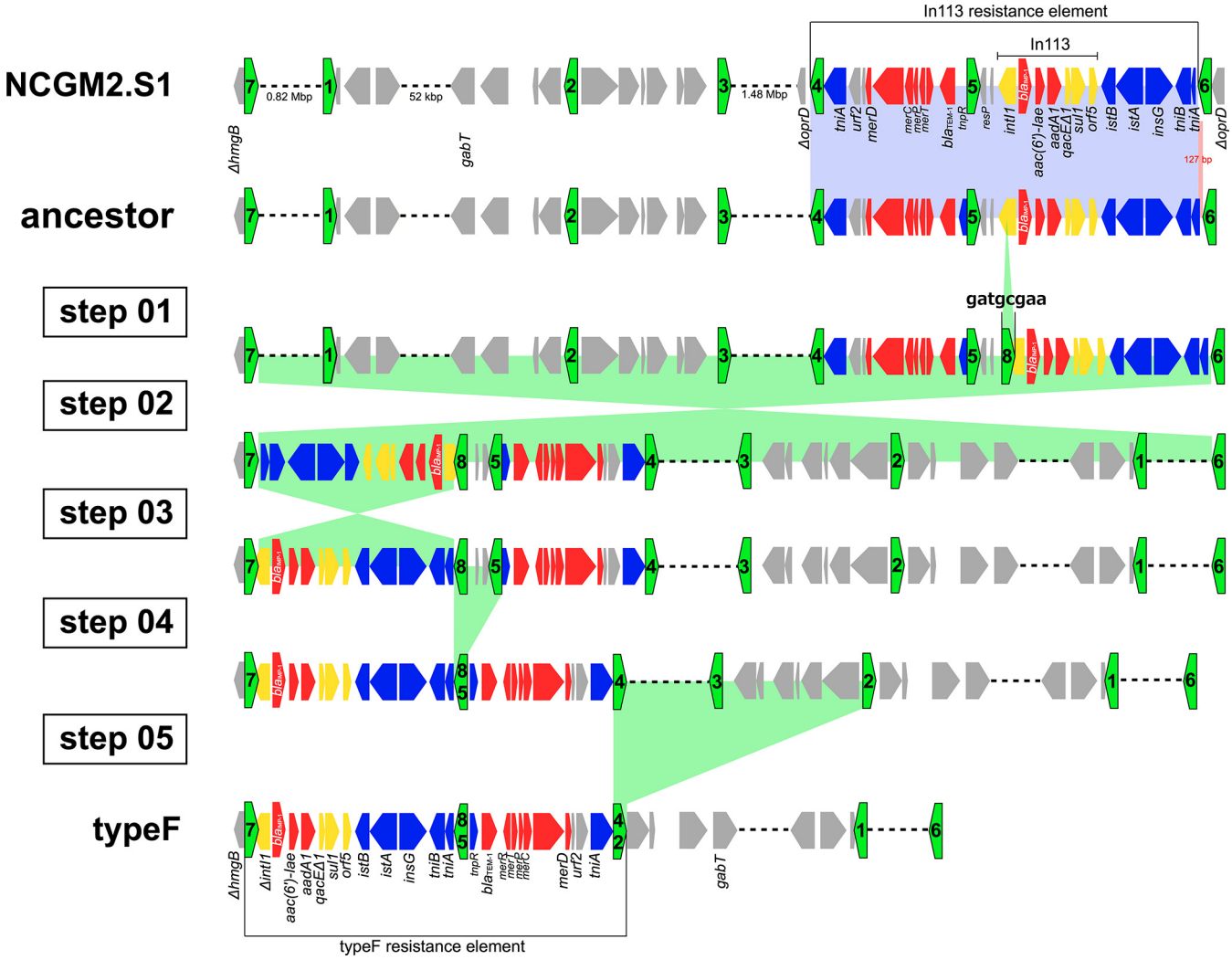


FIG 5 Hypothetical model of the genesis of *P. aeruginosa* PA058447 carrying the type F resistance element from an ancestor *P. aeruginosa* strain carrying the In113 resistance element. Green pentagons indicate IS*26*s and their orientations. Color shading indicates homologous regions. Coding sequences are shown as pentagons (green, IS*26*; blue, transposon; yellow, integron; red, antibiotic resistance gene; gray, other function or hypothetical protein). The In113 resistance element in an ancestor *P. aeruginosa* isolate is an IS*26*-transposon. The IS*26* transposon can be translocated into *oprD* to create NCGM2.S1. Genesis of *P. aeruginosa* PA058447 carrying the type F resistance element from the ancestor *P. aeruginosa* strain carrying the In113 resistance element can be achieved through IS*26*-8 insertion into *intI1* (step 01), genomic inversions (steps 02 and 03), and genomic deletions (steps 04 and 05) (the numbering of the steps does not imply a particular order).

complete genome sequence of NCGM2.S1 compared to the PAO1 genome depicted by BRIG (17). The data suggest that both genome sequences are very similar. In northern Hiroshima, prior to the abrupt increase of type F, a large outbreak of MDR *P. aeruginosa* carrying In113 (type E) occurred in 2003. We therefore postulate that type F originated from a similar MDR *P. aeruginosa* carrying In113 (i.e., type E) by IS*26* insertion into *intI1* gene and subsequent dynamic chromosomal rearrangements. In the NCGM2.S1 genome, seven IS*26*s (green pentagons) are present (Fig. 5). Three IS*26*s are within the In113-carrying resistance element inserted into *oprD*, and the other four are in other sites on the genome. Eight-base-pair target duplication flanking sequences in each IS*26* known as direct inverted repeats show a signature of history of mobility and integration mediated by IS*26* (22). In the NCGM2.S1 genome, the *hmgB* gene is disrupted by insertion of IS*26*-7 (green pentagon numbered 7 in Fig. 5), whose upstream sequence is identical to that of IS*26*L in the type F resistance element in PA058447 (Fig. 3B). Conversely, the downstream sequence of IS*26*-2 in NCGM2.S1 (Fig. 5) is identical to that of IS*26*R in the type F resistance element (Fig. 3B). Based on the information obtained by checking the identity of direct inverted-repeat 8-bp sequences flanking both sides of IS*26*s present in the type F resistance element and in NCGM2.S1 (data not shown), we assumed that the ancestor strain possessed the same framework of the genome as NCGM2.S1, but the In113 resistance element is present somewhere other than in the *oprD* (Fig. 5). We propose that several steps in genomic rearrangements through IS*26* insertion into *intI1* gene (step 01), IS*26*-mediated inversions (steps 02 and 03), and deletions (steps 04 and 05) in the ancestor genome may have generated PA058447 carrying the type F resistance element (Fig. 5). IS*26* has been implicated in disseminating resistance genes in many ways (19). Two IS*26*s in direct repeats forming a

transposon cause replicon fusion to transfer the transposon from the donor replicon to the target replicon (21, 23). Further, it appears to facilitate the mobilization of resistance genes on the chromosome. Mobility of the integrons by insertion of IS*26* into or proximally to the 5′- or 3′ conserved sequence (CS) is reported (24–30). For In53, insertion of IS*26* into *intI1* gene resulted in a knockout of the integrase function (30) as suggested in the type F resistance element. Further, multiple IS*26*s could contribute to formation of multiresistant loci by RecA-dependent homologous recombination. In this study, insertion of IS*26* into *intI1* gene probably facilitated the genomic rearrangement to generate the type F resistance element.

Type F and NCGM2.S1 belong to the same clonal lineage, ST235. ST235 is an international clone detected in Europe (31–41), Russia (42), Asia (7, 43–49), and the Middle East (50). ST235, ST111, and ST175 are regarded as epidemic high-risk clones, and ST235 lacks pyocyanin and pyoverdine production (51). The association of ST235 with a variety of carbapenem resistance acquired by horizontal transfer of the genes such as bla_{PER} (31, 36, 40, 41, 50), bla_{OXA} (36, 43, 50), bla_{GES} (31, 39), bla_{SPM} (36), bla_{VIM} (31–34, 36–38, 42, 44, 52), bla_{IMP} (36, 43–49), bla_{FIM} (53), and bla_{NDM} (54) suggests its importance in public health and in *P. aeruginosa*'s flexibility in adaptation. Recently, IMP-6-producing *P. aeruginosa* ST235 has become endemic in Korea (48). Bae et al. (43) reported the integron cassette structures of ST235, and one of them had a gene array identical to those of type F, except that the metallo-β-lactamase gene was bla_{IMP-6} instead of bla_{IMP-1}. Since IMP-6 differs from IMP-1 by only 1 amino acid at position 196 due to one nucleotide substitution, it may be reasonable to assume that they are derived from a common original integron structure. Further study may be necessary to delineate the evolutional relationship of these ST235s with very similar integron cassette structures. Of note, ST235 frequently shows multidrug resistance and the virulence genotype Δ*exoS exoU*$^+$ (where *exoS* is absent) (35), which is the case in type F and NCGM2.S1. The reason why MDR *P. aeruginosa* type F showed a higher dissemination potential for a long time in Hiroshima remains to be explained. Recently, Skurnik et al. (55) demonstrated that carbapenem-resistant *oprD* mutants show enhanced *in vivo* fitness through enhanced mucosal colonization potential, pathogenesis, and resistance to innate immunity. This is a supportive piece of evidence to explain the nationwide dissemination of NCGM2.S1, since it lacks functional OprD. On the other hand, MDR *P. aeruginosa* carrying the type F resistant element has an intact OprD. It should be noted that OprD is also acting as a specific channel for basic amino acids and small peptides (56, 57) and is possibly important for survival in the environment. Further molecular study is necessary to see how the bacteria increase their prevalence through the deployment of the cost-benefit selection of gene expression in infection process.

In conclusion, our longitudinal surveillance shows that a MDR *P. aeruginosa* strain carrying bla_{IMP-1} on an IS*26* transposon with a Tn*21* backbone became epidemic and showed continued persistence in Hiroshima for more than 9 years. We suggest that this clone originated from a *P. aeruginosa* isolate carrying In113 through an IS*26*-mediated insertional inactivation of the *intI1* gene and genomic reorganization.

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