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MECHANISMS OF RESISTANCE

Molecular Characterization of IMP-1-Producing *Enterobacter cloacae* Complex Isolates in Tokyo

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ABSTRACT Although KPC enzymes are most common among carbapenemases produced by *Enterobacter cloacae* complex globally, the epidemiology varies from one country to another. While previous studies have suggested that IMP enzymes are most common in Japan, detailed analysis has been scarce thus far. Here, we carried out a molecular epidemiological study and plasmid analysis of IMP-1-producing *E. cloacae* complex isolates collected from three hospitals in central Tokyo using whole-genome sequencing. Seventy-one isolates were classified into several sequence types (STs), and 49 isolates were identified as *Enterobacter hormaechei* ST78. Isolates of ST78 were divided into three clades by core-genome single nucleotide polymorphism (SNP)-based phylogenetic analysis. Whereas isolates of clade 3 were isolated from only one hospital, isolates of clade 1 and 2 were identified from multiple hospitals. Ten of 12 clade 1 isolates and 1 of 4 clade 2 isolates carried bla_{IMP-1} on IncHI2 plasmids, with high similarity of genetic structures. In addition, these plasmids shared backbone structures with IncHI2 plasmids carrying bla_{IMP} reported from other countries of the Asia-Pacific region. All isolates of clade 3 except one carried bla_{IMP-1} in In1426 on IncW plasmids. An isolate of clade 3, which lacked IncW plasmids, carried bla_{IMP-1} in In1426 on an IncFIB plasmid. These observations suggest that IMP-producing *E. cloacae* complex isolates with a diversity of host genomic backgrounds have spread in central Tokyo, and they indicate the possible contribution of IncHI2 plasmids toward this phenomenon.

KEYWORDS carbapenemase-producing *Enterobacteriaceae*, *Enterobacter cloacae* complex, IMP-1, metallo-β-lactamase, whole-genome sequencing, plasmid

Carbapenemase production is the most important mechanism contributing to carbapenem resistance in *Enterobacteriaceae*, and previous reports have suggested that carbapenemase-producing *Enterobacteriaceae* (CPE) have higher potential to cause lethal infection and nosocomial spread than non-carbapenemase-producing carbapenem-resistant *Enterobacteriaceae* (CRE) (1, 2). While KPC enzymes are the most common carbapenemases among those produced by *Enterobacteriaceae* on a global basis, the epidemiology varies from one country to another. Although information about the epidemiology of CPE in Japan has been limited, several studies have suggested that IMP enzymes are the most common among carbapenemases produced by *Enterobacteriaceae* (3–5).

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TABLE 1 ST and species of 71 IMP-1-producing *E. cloacae* complex isolates from three hospitals

ST	Species identification[a]	No. of isolates			
		Hospital A	Hospital B	Hospital C	Total
ST78	*E. hormaechei*	11	36	2	49
ST90	*E. hormaechei* subsp. *steigerwaltii*	8	0	0	8
ST175	*E. hormaechei* subsp. *steigerwaltii*	0	0	8	8
ST233	*E. hormaechei*	1	0	0	1
ST234	*E. hormaechei* subsp. *steigerwaltii*	0	1	0	1
ST242	*E. asburiae*	0	3	0	3
ST243	*E. kobei*	0	0	1	1

[a]Species identification was performed with ANI.

Molecular analysis revealed that several high-risk clones have contributed to the global spread of antimicrobial resistance genes in *Enterobacteriaceae*. For instance, *Escherichia coli* sequence type 131 (ST131) and *Klebsiella pneumoniae* ST258 have played crucial roles in the global spread of $bla_{\text{CTX-M}}$ and bla_{KPC}, respectively (6). Although the presence of several high-risk clones such as ST78 and ST66 among *Enterobacter cloacae* complex (ECC) isolates has been suggested, information about the relationship between these high-risk clones and carbapenemase genes has been limited until now (7, 8).

Plasmids carrying resistance genes play an important role in the spread of resistance genes among different clones and different species. In fact, institutional and regional outbreaks of CPE involving multiple clones and/or species mediated by specific plasmids carrying carbapenemase genes have been reported repeatedly (9–11). This underscores the importance of detailed analysis of resistance plasmids in molecular epidemiological studies of CPE. Although complete nucleotide sequences of numerous plasmids carrying carbapenemase genes, such as bla_{KPC}, bla_{NDM}, and $bla_{\text{OXA-48-like}}$, have been determined (12–15), detailed information about the plasmids carrying bla_{IMP} has been scarce partly because CPE producing IMP enzymes have been rare in most parts of the world (8).

In this study, we performed molecular epidemiological analysis of metallo-β-lactamase (MBL)-producing ECC isolates collected from three hospitals in central Tokyo and characterized plasmids harboring bla_{IMP}, with a special emphasis on the isolates belonging to ST78.

RESULTS

Identification of MBL genes and whole-genome sequencing of MBL-producing ECC isolates. A total of 71 frozen stored MBL-producing ECC clinical isolates, consisting of 20 isolates from hospital A, 40 isolates from hospital B, and 11 isolates from hospital C, were collected (Table 1). All isolates were positive for $bla_{\text{IMP-1-group}}$ by PCR. We sequenced the whole genomes of all MBL-producing ECC isolates. A total of 71 genome sequences were obtained at an average depth of 149.3 (standard deviation [SD], 48.1) (see Data Set S1 in the supplemental material). Assembled genomes had an average number of 179.1 (SD, 70.4) contigs and an N_{50} value of 90,921 bp (SD, 44,765.7 bp).

MLST and species identification. Seventy-one ECC isolates were classified into 7 STs by multilocus sequence typing (MLST) (Table 1). Isolates belonging to ST78 were most common, accounting for 69.0% ($n = 49$) of all isolates, followed by those belonging to ST90 ($n = 8$) and ST175 ($n = 8$). The isolates belonging to ST90, ST175, and ST234 were identified as *Enterobacter hormaechei* subsp. *steigerwaltii*, and the isolates belonging to ST242 and ST243 were identified as belonging to *Enterobacter asburiae* and *Enterobacter kobei*, respectively, by average nucleotide identity (ANI). The isolates belonging to ST78 and ST233 were identified as *E. hormaechei*. While the isolate belonging to ST78 was detected in all three hospitals, the isolates belonging to other STs were collected only from a specific hospital: ST90 and ST233 from hospital A, ST242 and ST234 from hospital B, and ST175 and ST243 from hospital C.

Phylogenetic analysis and estimation of evolutionary rate. Core-genome single nucleotide polymorphism (SNP)-based phylogenetic analysis revealed the presence of

FIG 1 Phylogenetic tree of IMP-1-producing *Enterobacter hormaechei* ST78 isolates ($n = 49$; TUM prefixes have been removed for clarity) constructed with maximum-likelihood phylogenetic analysis based on single nucleotide polymorphisms (SNPs) in the core genome and excluding homologous recombination sequences. The core-genome region was 86.9% (4,027,037/4,633,407 bp) of the genome of the reference strain, *E. cloacae* ECNIH3 ST97. The scale distance corresponds to the number of substitutions per site.

three major clades of MBL-producing *E. hormaechei* ST78 isolates. Clades 1, 2, and 3 consisted of 12, 4, and 33 isolates, respectively (Fig. 1). While isolates of clade 3 were isolated only from hospital B, isolates belonging to clade 1 and clade 2 were isolated from multiple hospitals (Table 2). The average substitution rate in the core genome was estimated to be 4.53 SNPs (95% highest posterior density interval, 1.40 to 7.74 SNPs) per genome per year, and the time of divergence of clade 1 and clade 2 was estimated to be around 120 years ago (Fig. S1).

Antimicrobial susceptibility and resistance genes. All MBL-producing *E. hormaechei* ST78 isolates were resistant to cefotaxime and moxalactam and susceptible to amikacin. The rates of resistance to piperacillin, piperacillin-tazobactam, cefepime, aztreonam, imipenem, and ciprofloxacin were 26.5% ($n = 13$), 26.5% ($n = 13$), 59.2% ($n = 29$), 51.0% ($n = 25$), 18.4% ($n = 9$), and 55.1% ($n = 28$), respectively (Table 2).

All isolates of clade 1 and clade 2 harbored bla_{IMP-1}, *aac(6′)-Ilc*, *sul1*, and *tet(B)*. An isolate of clade 2 (TUM11137) also carried *dfrA15*. All isolates of clade 3 harbored bla_{IMP-1} and *aac(6′)-Ib-cr*. In addition, *dfrA*, *strA*, *strB*, *qnaS1*, and *sul1* were carried by 29, 22, 22, 17, and 2 isolates of clade 3, respectively (Table 2).

Characteristics of the plasmids harboring bla_{IMP-1}. While replicon typing with PlasmidFinder confirmed that all isolates of clades 1 and 2 carried IncHI2 plasmids, all isolates of clade 3 except one (TUM10695) carried IncW plasmids (Table 2). bla_{IMP-1} and *rep* genes of IncW were found on the same contig in 17 isolates of clade 3. Most of the isolates of clade 3 also carried an IncFIB and/or IncFII plasmid. TUM10695 carried an

TABLE 2 Characteristics of IMP-1-producing *Enterobacter hormaechei* ST78

Clade and isolate no.	Hospital	Isolation date (mo/day/yr)	Antimicrobial resistance genes	Plasmid replicon(s)	MIC (μg/ml)								
					Imipenem	Cefotaxime	Cefepime	Aztreonam	Piperacillin-tazobactam	Piperacillin	Moxalactam	Amikacin	Ciprofloxacin
Clade 1													
TUM10689	B	8/24/2010	bla_{IMP-1}, *aac(6′)-Ilc*, *sul1*, *tet*(B)	IncHI2	0.5	512	32	128	256/4	256	>512	1	0.25
TUM10699	B	9/16/2010	bla_{IMP-1}, *aac(6′)-Ilc*, *sul1*, *tet*(B)	IncHI2	1	512	32	128	>512/4	>512	>512	2	0.25
TUM11041	A	1/19/2007	bla_{IMP-1}, *aac(6′)-Ilc*, *sul1*, *tet*(B)	IncHI2	≥0.25	512	64	64	128/4	128	>512	2	0.125
TUM11042	A	2/24/2007	bla_{IMP-1}, *aac(6′)-Ilc*, *sul1*, *tet*(B)	IncHI2	≥0.25	>512	64	256	512/4	512	>512	2	0.125
TUM11043	A	3/23/2007	bla_{IMP-1}, *aac(6′)-Ilc*, *sul1*, *tet*(B)	IncHI2	≥0.25	>512	64	256	256/4	256	>512	1	0.25
TUM11045	A	9/18/2007	bla_{IMP-1}, *aac(6′)-Ilc*, *sul1*, *tet*(B)	IncHI2	4	>512	256	128	512/4	512	>512	2	0.25
TUM11046	A	7/7/2009	bla_{IMP-1}, *aac(6′)-Ilc*, *sul1*, *tet*(B)	IncHI2	2	>512	64	256	>512/4	>512	>512	2	0.25
TUM11047	A	7/9/2009	bla_{IMP-1}, *aac(6′)-Ilc*, *sul1*, *tet*(B)	IncHI2	2	>512	64	256	512/4	512	>512	2	0.25
TUM11048	A	7/21/2009	bla_{IMP-1}, *aac(6′)-Ilc*, *sul1*, *tet*(B)	IncHI2	2	>512	64	256	512/4	512	>512	2	0.5
TUM11049	A	7/24/2009	bla_{IMP-1}, *aac(6′)-Ilc*, *sul1*, *tet*(B)	IncHI2	1	>512	64	128	64/4	64	>512	2	0.25
TUM11050	A	9/9/2009	bla_{IMP-1}, *aac(6′)-Ilc*, *sul1*, *tet(B)*	IncHI2	2	>512	128	256	512/4	512	>512	2	0.25
TUM11053	A	5/26/2010	bla_{IMP-1}, *aac(6′)-Ilc*, *sul1*, *tet*(B)	IncHI2	4	>512	128	256	256/4	256	>512	2	0.25
Clade 2													
TUM10671	B	7/23/2010	bla_{IMP-1}, *aac(6′)-Ilc*, *sul1*, *tet*(B)	IncHI2	4	256	32	32	64/4	64	>512	2	1
TUM11044	A	8/10/2007	bla_{IMP-1}, *aac(6′)-Ilc*, *sul1*, *tet*(B)	IncHI2	2	256	128	32	32/4	32	>512	2	2
TUM11136	C	9/22/2009	bla_{IMP-1}, *aac(6′)-Ilc*, *sul1*, *tet*(B)	IncHI2	4	512	32	32	128/4	128	>512	1	1
TUM11137	C	10/25/2009	bla_{IMP-1}, *aac(6′)-Ilc*, *sul1*, *tet*(B), *dfrA25*	IncHI2, IncN	64	128	512	8	32/4	32	>512	1	2
Clade 3													
TUM10660	B	6/24/2010	bla_{IMP-1}, *aac(6′)-Ib*, *dfrA15*	IncW, IncFII, IncFIB	4	256	64	0.5	16/4	16	>512	4	2
TUM10661	B	6/24/2010	bla_{IMP-1}, *aac(6′)-Ib*	IncW, IncFII, IncFIB	16	>512	64	16	64/4	64	>512	4	16
TUM10662	B	7/5/2010	bla_{IMP-1}, *aac(6′)-Ib*, *strAB*, *qnrS1*	IncW, IncFII	1	64	8	64	16/4	16	256	2	32
TUM10663	B	7/5/2010	bla_{IMP-1}, *aac(6′)-Ib*, *dfrA15*, *strAB*	IncW, IncFII	0.5	64	8	< = 0.25	16/4	16	256	2	64

(Continued on next page)

TABLE 2 (Continued)

Clade and isolate no.	Hospital	Isolation date (mo/day/yr)	Antimicrobial resistance genes	Plasmid replicon(s)	MIC (μg/ml)								
					Imipenem	Cefotaxime	Cefepime	Aztreonam	Piperacillin-tazobactam	Piperacillin	Moxalactam	Amikacin	Ciprofloxacin
TUM10664	B	7/6/2010	bla_{IMP-1}, *aac(6′)-Ib*, *dfrA15*, *strAB*, *qnrS1*	IncW, IncFII	2	>512	128	64	64/4	64	>512	4	8
TUM10665	B	7/7/2010	bla_{IMP-1}, *aac(6′)-Ib*, *dfrA15*, *strAB*	IncW	0.5	512	8	64	128/4	128	>512	2	64
TUM10666	B	7/9/2010	bla_{IMP-1}, *aac(6′)-Ib*, *dfrA15*, *strAB*, *qnrS1*	IncW, IncFII	1	256	16	1	16/4	16	512	8	32
TUM10667	B	7/10/2010	bla_{IMP-1}, *aac(6′)-Ib*, *dfrA15*, *strAB*, *qnrS1*	IncW, IncFII, IncFIB	0.5	32	4	≥0.25	8/4	8	256	4	16
TUM10669	B	5/6/2010	bla_{IMP-1}, *aac(6′)-Ib*, *dfrA15*, *strAB*	IncW, IncFII	0.5	64	4	≥0.25	8/4	8	>512	2	32
TUM10670	B	7/14/2010	bla_{IMP-1}, *aac(6′)-Ib*, *dfrA15*, *strAB*, *qnrS1*	IncW, IncFII	1	64	8	2	8/4	8	128	2	32
TUM10672	B	7/26/2010	bla_{IMP-1}, *aac(6′)-Ib*, *dfrA15*	IncW, IncFII, IncFIB	0.5	64	4	16	4/4	4	128	4	2
TUM10673	B	7/31/2010	bla_{IMP-1}, *aac(6′)-Ib*, *dfrA15*, *strAB*, *qnrS1*	IncW, IncFII	2	128	16	1	16/4	16	>512	8	32
TUM10674	B	8/9/2010	bla_{IMP-1}, *aac(6′)-Ib*, *dfrA15*	IncW, IncFII, IncFIB	0.5	32	8	4	4/4	4	128	4	2
TUM10675	B	8/10/2010	bla_{IMP-1}, *aac(6′)-Ib*, *dfrA15*	IncW, IncFII, IncFIB	0.5	32	4	< = 0.25	4/4	4	128	4	4
TUM10676	B	8/11/2010	bla_{IMP-1}, *aac(6′)-Ib*, *dfrA15*, *strAB*, *qnrS1*	IncW, IncFII	2	64	8	1	8/4	8	>512	8	64
TUM10677	B	8/12/2010	bla_{IMP-1}, *aac(6′)-Ib*, *dfrA15*	IncW, IncFII, IncFIB	2	128	32	0.5	8/4	8	>512	8	2
TUM10678	B	8/12/2010	bla_{IMP-1}, *aac(6′)-Ib*, *dfrA15*, *strAB*, *qnrS1*	IncW, IncFII	1	128	4	0.5	4/4	4	128	2	32
TUM10679	B	8/12/2010	bla_{IMP-1}, *aac(6′)-Ib*, *dfrA15*, *strAB*, *qnrS1*	IncW	1	64	8	4	8/4	8	256	2	64
TUM10680	B	8/17/2010	bla_{IMP-1}, *aac(6′)-Ib*, *strAB*, *qnrS1*	IncW, IncFII	2	256	64	< = 0.25	8/4	8	>512	8	16
TUM10683	B	8/18/2010	bla_{IMP-1}, *aac(6′)-Ib*, *dfrA15*	IncW, IncFII, IncFIB	0.5	32	8	0.5	4/4	4	128	8	4
TUM10684	B	8/19/2010	bla_{IMP-1}, *aac(6′)-Ib*, *sul1*, *dfrA15*, *strAB*, *qnrS1*	IncW, IncFII	0.5	64	16	1	16/4	16	>512	8	64

(Continued on next page)

TABLE 2 (Continued)

Clade and isolate no.	Hospital	Isolation date (mo/day/yr)	Antimicrobial resistance genes	Plasmid replicon(s)	MIC (μg/ml)								
					Imipenem	Cefotaxime	Cefepime	Aztreonam	Piperacillin-tazobactam	Piperacillin	Moxalactam	Amikacin	Ciprofloxacin
TUM10685	B	8/19/2010	bla_{IMP-1}, *aac(6′)-Ib*, *dfrA15*, *strAB*, *qnrS1*	IncW, IncFII	1	128	8	64	8/4	8	256	2	64
TUM10686	B	8/23/2010	bla_{IMP-1}, *aac(6′)-Ib*, *dfrA15*	IncW, IncFII, IncFIB	0.5	128	4	16	4/4	4	256	2	4
TUM10687	B	8/23/2010	bla_{IMP-1}, *aac(6′)-Ib*, *dfrA15*	IncW, IncFII, IncFIB	0.5	256	4	32	4/4	4	128	2	2
TUM10690	B	8/24/2010	bla_{IMP-1}, *aac(6′)-Ib*, *sul1*, *dfrA15*, *strAB*, *qnrS1*	IncW, IncFII	0.5	64	4	2	8/4	8	256	4	16
TUM10691	B	8/24/2010	bla_{IMP-1}, *aac(6′)-Ib*, *dfrA15*, *strAB*, *qnrS1*	IncW	1	32	8	0.5	8/4	8	256	2	64
TUM10692	B	8/24/2010	bla_{IMP-1}, *aac(6′)-Ib*, *dfrA15*, *strAB*, *qnrS1*	IncW	1	64	16	0.5	8/4	8	>512	4	64
TUM10693	B	8/30/2010	bla_{IMP-1}, *aac(6′)-Ib*, *dfrA15*, *strAB*	IncW	4	256	128	1	32/4	32	>512	8	4
TUM10694	B	8/30/2010	bla_{IMP-1}, *aac(6′)-Ib*, *strAB*	IncW	4	256	128	0.5	32/4	32	>512	8	8
TUM10695	B	9/1/2010	bla_{IMP-1}, *aac(6′)-Ib*, *strAB*, *qnrS1*	IncFII, IncFIB	0.5	32	2	<= 0.25	8/4	8	64	4	32
TUM10696	B	9/7/2010	bla_{IMP-1}, *aac(6′)-Ib*, *dfrA15*, *strAB*, *qnrS1*	IncW, IncFII	0.5	128	4	64	16/4	16	128	2	2
TUM10697	B	9/11/2010	bla_{IMP-1}, *aac(6′)-Ib*, *dfrA15*	IncW, IncFII, IncFIB	1	64	16	8	64/4	64	>512	4	32
TUM10698	B	9/11/2010	bla_{IMP-1}, *aac(6′)-Ib*, *dfrA15*	IncW, IncFII, IncFIB	2	128	16	64	8/4	8	>512	4	4

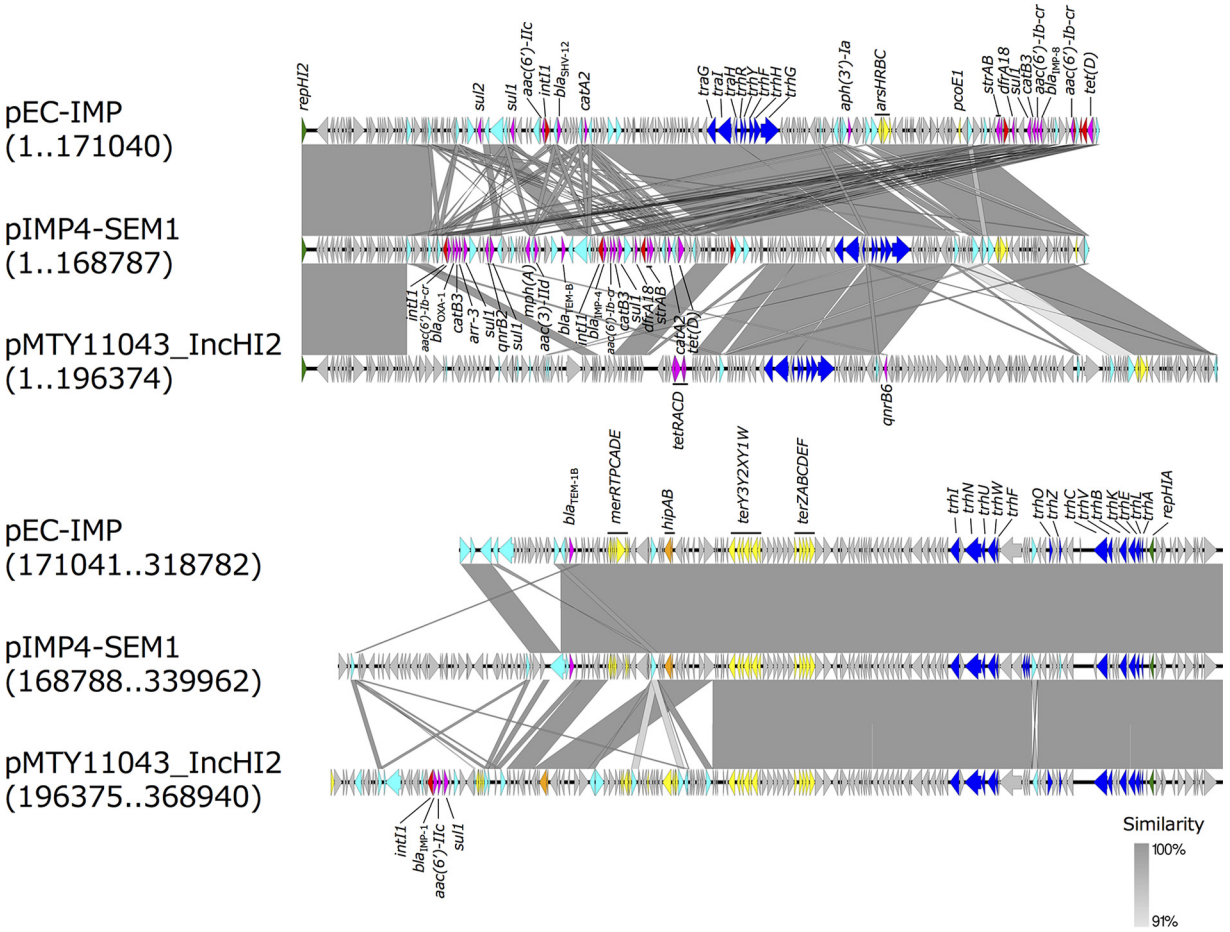

FIG 2 Comparison of pMTY11043_IncHI2 (GenBank accession number AP018352.1) carrying bla_{IMP-1} with pEC-IMP (GenBank accession number EU855787) and pIMP4-SEM (KX810825) drawn with EasyFig, version 2.1. These IncHI2 plasmids belong to pMLST-ST1. Block arrows indicate confirmed or putative open reading frames (ORFs) and their orientations. Arrow size is proportional to the predicted ORF length. The color code is as follows: green, replication initiation protein genes; blue, conjugal transfer genes; cyan, transposase genes; yellow, heavy metal resistance genes; red, integrase genes; magenta, antibiotic resistance genes; orange, toxin-antitoxin system genes. Putative, hypothetical, and unknown genes are represented by gray arrows.

IncFIB plasmid and IncFII plasmid, and bla_{IMP-1} and *rep* genes of IncFIB were found on the same contig.

Plasmids carrying bla_{IMP-1} were successfully transferred by conjugation in 10 of 12 clade 1 isolates (except TUM10689 and TUM10699), 2 of 4 clade 2 isolates (TUM11136 and TUM11137), and 31 of 33 clade 3 isolates (except TUM10695 and 10698). All transconjugants of clade 1 isolates and one of two transconjugants of clade 2 isolates (TUM11136) were positive for IncHI2 by PCR-based Inc/Rep typing. Transconjugants of TUM11137 were positive for IncN. All transconjugants of clade 3 isolates were positive for IncW.

A representative plasmid of IncHI2, pMTY11043_IncHI2, from TUM11043 was 368,940 bp in length, exhibited a GC content of 47.3%, and had 425 predicted open reading frames (ORFs) (GenBank accession number AP018352.1). It carried a class 1 integron containing bla_{IMP-1} [In316 with bla_{IMP-1}, *aac(6′)-IIc*, and *sul1*] and *qnrB6*. The backbone structure of pMTY11043_IncHI2 was highly similar to that of pIMP4-SEM1 of IMP-4-producing *Salmonella enterica* serovar Typhimurium (GenBank accession number KX810825) and pEC-IMP of IMP-8-producing *E. cloacae* (EU855787) (Fig. 2) (16, 17). pMTY11043_IncHI2, pIMP4-SEM1, and pEC-IMP carried genes for the HipBA toxin-antitoxin system (*hipBA*) and heavy metal ion resistance genes for arsenic (*arsHRBC* operon) and tellurium (*terABCDEFWXYZ*). Of the 11 IncHI2 plasmids carrying bla_{IMP-1}, which were successfully transferred by conjugation, 7 were determined to be ST1 by *in*

TABLE 3 Results of pMLST of conjugative IncHI2 plasmids harboring bla_{IMP-1} carried by the isolates of clades 1 and 2

Clade and plasmid	Host	Allele profile		pMLST result
		smr0199	*smr0018*	
Clade 1				
pMTY11041_IncHI2	TUM11041	1	1	ST1
pMTY11042_IncHI2	TUM11042	1	1	ST1
pMTY11043_IncHI2	TUM11043	1	1	ST1
pMTY11045_IncHI2	TUM11045	1	1	ST1
pMTY11046_IncHI2	TUM11046	1	1	ST1
pMTY11047_IncHI2	TUM11047	1	1	ST1
pMTY11048_IncHI2	TUM11048	1	One nucleotide substitution compared with allele 1[b]	ND[a]
pMTY11049_IncHI2	TUM11049	1	Seven nucleotide substitutions compared with allele 5[c]	ND
pMTY11050_IncHI2	TUM11050	1	1	ST1
pMTY11053_IncHI2	TUM11053	1	Complete nucleotide sequence of allele 1 disrupted by IS*Vsa5*	ND
Clade 2				
pMTY11136_IncHI2	TUM11136	1	1	ST1

[a]ND, not determined.
[b]*smr0018* sequence of pMTY11048_IncHI2 is the closest match to allele 1.
[c]*smr0018* sequence of pMTY11049_IncHI2 is the closest match to allele 5.

silico plasmid MLST (pMLST) (Table 3). The nucleotide sequences of conjugative IncHI2 plasmids carrying bla_{IMP-1} from isolates of clade 1 and 2 had high similarity (Fig. S2).

A representative plasmid of IncW, pMTY10660_IncW (GenBank accession number AP018350.1), was 33,748 bp in length, exhibited a GC content of 57.2%, and had 40 predicted ORFs. It carried a class 1 integron containing bla_{IMP-1} [In1426 with bla_{IMP-1} and *aac(6′)-Ib-cr*]. The nucleotide sequence of pMTY10660_IncW highly resembled that of pR388 from *E. coli* with trimethoprim-sulfonamide resistance (GenBank accession number BR000038) except for the contents of gene cassettes of a class 1 integron (Fig. 3).

pMTY10695_IncFIB (GenBank accession number AP018351.1) was 112,622 bp in length, exhibited a GC content of 51.8%, and had 134 predicted ORFs. Large numbers of coding sequences (CDSs) were annotated as hypothetical proteins of unknown function using the DFAST automatic annotation pipeline. pMTY10695_IncFIB carried In1426-like pMTY10660_IncW. In1426 was located between the inverted repeat, left (IRL), and inverted repeat, right (IRR), of IS*Pa38* together with a curtailed *tnpA* of IS*Pa38* lacking the central part of the gene in pMTY10695_IncFIB (Fig. 4). The backbone structure of IncFIB plasmids from isolates of clade 3 had high similarity, except that In1426 and its surrounding structure were inserted only in pMTY10695_IncFIB (Fig. S3).

DISCUSSION

In this study, we analyzed 71 MBL-producing ECC isolates collected from three hospitals in central Tokyo, Japan. Although all isolates carried bla_{IMP-1}, MLST revealed

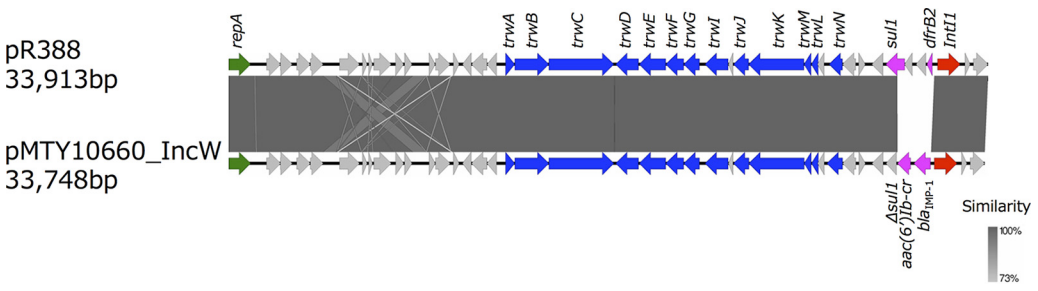

FIG 3 Comparison of the nucleotide sequences of pMTY10660_IncW harboring bla_{IMP-1} (GenBank accession number AP018350.1) and pR388 (BR000038). The color code is the same as that described in the legend of Fig. 2.

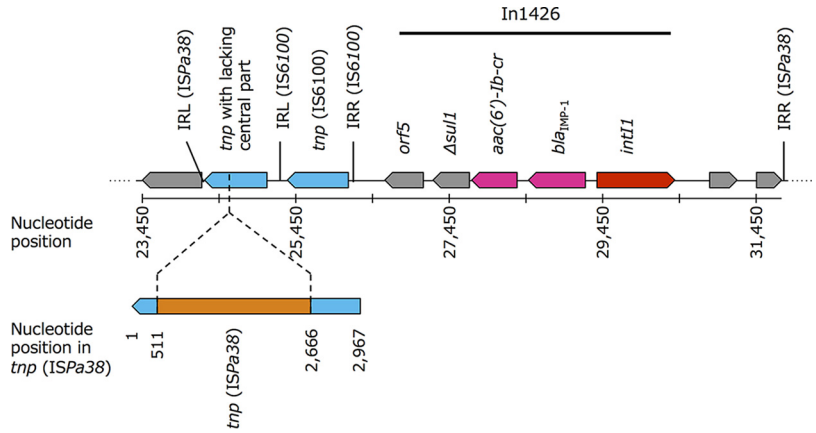

FIG 4 Structural features of In1426, a class 1 integron containing bla_{IMP-1}, and surrounding nucleotide sequences in pMTY10695_IncFIB (GenBank accession number AP018351.1). The insertion sequence IS*6100* was located downstream of In1426. The central part of the transposase gene of IS*Pa38* (2,154 bp/2,967 bp) was lacking. The missing region is in orange. IRR, inverted repeat, right; IRL, inverted repeat, left.

that isolates of various STs contributed to the regional spread of IMP-1-producing ECC. ST78 was most common and the only clone isolated from all three hospitals. Isolates of *E. hormaechei* ST78 were classified into three clades by core-genome SNP-based phylogenetic analysis. Plasmids of four Inc types, IncHI2, IncW, IncN, and IncFIB, were involved in the spread of bla_{IMP-1}.

The MLST scheme for ECC was developed by Miyoshi-Akiyama et al. (18). In that study, 101 ECC isolates collected from a hospital in Tokyo and a commercial clinical laboratory in Japan were classified by MLST, and isolates of ST78 were most common (11.9%). While criteria for the collection of the isolates in the study were not described in detail, it is possible that *E. hormaechei* ST78 is dominant among ECC clinical isolates in Japan regardless of their antibiotic susceptibilities. Our study showed that the proportion of the isolates of *E. hormaechei* ST78 was extremely high among IMP-1-producing ECC isolates collected in central Tokyo. ST78 was also one of the most common clones among the expanded-spectrum cephalosporin-resistant ECC isolates collected across Europe and Israel in a previous study (7). Isolates of *E. hormaechei* ST78 may have high potential to acquire genes for resistance to extended-spectrum β-lactams, as has been observed in *E. coli* ST131 and *K. pneumoniae* ST258.

While isolates of clade 3 of *E. hormaechei* ST78 were detected only from hospital B, isolates of clades 1 and 2 were detected from multiple hospitals. It would be unusual for a patient to visit more than one of the three hospitals participating in this study because they cover different populations. In addition, calculation of the nucleotide substitution rate revealed that clade 1 and clade 2 branched clearly in advance of the period of the isolation of the IMP-1-producing ECC isolates in this study. Therefore, the isolation of IMP-1-producing ECC isolates of the same clade in different hospitals may suggest the endemicity of clades 1 and 2 in this area. It appears that clonal spread of isolates of clade 3 was occurring in hospital B. On the other hand, isolates of clades 1 and 2 were also detected in hospital B during the 5-month period for the collection of the isolates. A similar situation was observed in an institution-wide outbreak of KPC-producing *K. pneumoniae* (9). Nosocomial spread of a specific outbreak strain (*K. pneumoniae* ST941) and sporadic influx of an endemic strain (*K. pneumoniae* ST258) took place simultaneously. In addition, groups of IMP-1-producing ECC isolates belonging to the same ST were recovered in all three hospitals: 8 isolates of *E. hormaechei* subsp. *steigerwaltii* ST90 in hospital A, 3 isolates of *E. asburiae* ST242 in hospital B, and 8 isolates of *E. hormaechei* subsp. *steigerwaltii* ST175 from hospital C together with isolates of *E. hormaechei* ST78. While isolates of *E. hormaechei* subsp. *steigerwaltii* ST175 from hospital C were divided into 2 groups by pulsed-field gel electrophoresis (PFGE),

isolates of *E. hormaechei* subsp. *steigerwaltii* ST90 from hospital A were indistinguishable, and the same applied to isolates of *E. asburiae* ST242 from hospital B (data not shown). A polyclonal outbreak of KPC-producing ECC was also reported in another study (19). These findings may reflect the complexity of the molecular epidemiology of outbreaks caused by CPE.

All isolates of clades 1 and 2 of *E. hormaechei* ST78 carried highly similar IncHI2 plasmids harboring bla_{IMP-1} that were successfully transferred by conjugation even though clades 1 and 2 appeared to have diverged long ago, as mentioned above. We applied the PacBio RS system to obtain the complete sequence of a representative IncHI2 plasmid (pMTY11043_IncHI2). Detailed plasmid analysis using technologies which generate long-read data, including the PacBio RS system, has provided important information, such as the evidence for transfer of plasmids carrying carbapenemase genes in the hospital environment and complex plasmid rearrangement during long-term human colonization of CPE in previous studies (12, 20). The whole nucleotide sequence of pMTY11043_IncHI2 demonstrated that the backbone structure of the plasmid had high similarity with that of pEC-IMP harboring bla_{IMP-8} of an ECC clinical isolate from Taiwan and pIMP4-SEM1 harboring bla_{IMP-4} of *S.* Typhimurium from cats in Australia (16, 17). Furthermore, these IncHI2 plasmids have common genes for toxin-antitoxin systems and heavy metal resistance. Carriage of these genes may aid the consequential maintenance of the plasmids in diverse environments (21–23). Interestingly, it has been demonstrated that transfer of IncHI2 plasmids was involved in the spread of bla_{IMP-4} between different species of *Enterobacteriaceae* in previous studies from Queensland in Australia (10). These observations, together with our findings, suggest that IncHI2 may play a key role in the spread of bla_{IMP} in *Enterobacteriaceae* although it remains to be elucidated whether the impact is regional or global.

Clade 3 of *E. hormaechei* ST78 carrying IncW plasmids harboring bla_{IMP-1} has been identified as an institutional outbreak clone of hospital B. Interestingly, an isolate of clade 3 from hospital B (TUM10695) lacked an IncW plasmid and carried an IncFIB plasmid harboring In1426, possibly transferred from IncW plasmids by IS*Pa38*-mediated transposition.

Our study has several limitations. First, the number of hospitals participating in the collection of the isolates was limited, and the hospitals represented only the central area of Tokyo. Whether the epidemiology of these hospitals reflects that throughout Japan is unknown. Nevertheless, the complexity of the molecular epidemiology of the hospital-wide outbreak and the regional spread of MBL-producing ECC were demonstrated. Second, we did not collect clinical information of the patients from whom the MBL-producing ECC isolates were isolated. Active surveillance cultures might have been taken in outbreak settings together with cultures from the patients with infections. In addition, the duration of the collection of the isolates was different in each hospital. Therefore, interpretation of the significance of the number of isolates belonging to each clone is difficult.

In this study, we demonstrated that *E. hormaechei* ST78 was the major clone among IMP-1-producing ECC in central Tokyo. While IMP-1-producing *E. hormaechei* ST78 was divided into three clades by core-genome SNP-based phylogenetic analysis, isolates of clades 1 and 2 were isolated from multiple hospitals. Four types of plasmids were involved in the spread of bla_{IMP-1} in central Tokyo, and IncHI2 plasmids carried by isolates of clades 1 and 2 had backbone structures similar to the structure of an IncHI2 plasmid carrying bla_{IMP} reported from other countries of the Asian-Pacific region.

MATERIALS AND METHODS

Collection of bacterial isolates. Frozen stored MBL-producing ECC clinical isolates were sent to the Department of Microbiology and Infectious Diseases, School of Medicine, Toho University, from three university hospitals with >900 beds engaged in tertiary medical care in central Tokyo, Japan. Primary identification of bacterial species was performed with a Microscan WalkAway (Beckman Coulter, CA, USA) or Vitek2 (bioMérieux, France) instrument, and MICs were determined with Microscan WalkAway or the broth dilution method, according to CLSI guidelines, with Eiken dry plates (Eiken Chemical Co., Ltd. Tokyo, Japan) in each case (24). ECC isolates showing MICs of 4 μg/ml or higher for ceftazidime or ceftizoxime were judged as possible MBL producers, and confirmation testing was performed with

ceftazidime 30-μg disks (Eiken Chemical, Tokyo, Japan) and sodium mercaptoacetate (SMA) 3-mg disks (Eiken Chemical, Tokyo, Japan). ECC isolates showing enlargement of inhibitory zone diameters around the ceftazidime disk by >5 mm when it was located adjacent to an SMA disk were determined to be MBL-producing ECC (24). These isolates were stored frozen from January 2007 to February 2011 in hospital A, from May 2010 to September 2010 in hospital B, and from July 2008 to October 2010 in hospital C.

This study was conducted with approval from the Research Ethics Board of the Toho University School of Medicine (no. 25068).

Screening of *bla*$_{\text{IMP-1-group}}$ genes by PCR. PCR screening of *bla*$_{\text{IMP-1-group}}$ genes of MBL-producing ECC isolates was performed as reported previously (5).

Whole-genome sequencing, identification of bacterial species, and MLST. To determine the draft whole-genome sequence of MBL-producing ECC isolates, DNA was extracted from bacteria by phenol-chloroform treatment. We used a Nextera XT DNA library preparation kit (Illumina, Inc., CA, USA) to prepare DNA libraries for sequencing. Libraries were sequenced on a MiSeq system for 600 cycles (300-bp paired-end reads). Draft genomes (contigs) were obtained using the CLC Genomics Workbench (Qiagen). Species identification was performed using average nucleotide identity (ANI) and the EzBioCloud database (25, 26). We used cutoff values of 96% (27, 28) or more and 98% or more of the ANI value compared with the genomic sequences of the type strain for species and subspecies identification, respectively. The sequence type was confirmed using BLASTn-based *in silico* MLST with *de novo*-assembled whole-genome sequencing data using *E. cloacae* MLST databases in PubMLST.org (http://pubmlst.org/ecloacae/) (29).

Forty-nine isolates identified as IMP-1-producing *Enterobacter hormaechei* ST78 were used for additional analysis as described below.

Phylogenetic analysis and estimation of evolutionary rate. Core-genome single nucleotide polymorphism (SNP)-based phylogenetic analysis was performed with whole-genome sequencing data. The MiSeq sequencing data were aligned to the genomic sequence of the reference isolate, *E. cloacae* ECNIH3 ST93 (GenBank accession number CP008897), using the Burrows-Wheeler Aligner (BWA) with "MEM" option (30). The *E. cloacae* ECNIH3 ST97 isolate was selected as the reference isolate for mapping because it was closest to ST78 by the phylogenetic analysis based on seven MLST allele sequences (3,511 bp) among isolates with available whole-genome data sets. We constructed a core-genome alignment using SAMtools (version 1.1) mpileup (31) and VarScan (version 2.3.7) mpileup2cns (32) and then a maximum-likelihood tree using PhyML (33). Using this as the starting tree, we inferred homologous recombination events that imported DNA fragments from outside the ST and constructed a clonal phylogeny with corrected branch lengths using ClonalFrameML (34). We also conducted this analysis after excluding all of clade 3 (Fig. 1) consisting of an institutional outbreak clone in a single hospital in 2010 that seemed to evolve differently from the other strains. After excluding the recombined sequences, we estimated the average substitution rate across all sites in the core genome and the divergence time of clade 1 and clade 2 identified by the phylogenetic analysis using BEAST (version 2.4.7) (35). BEAST was run for 20 million generations, sampling every 200 states, using the general time-reversible (GTR) substitution model.

Antibiotic susceptibility testing and identification of antimicrobial resistance genes. Antibiotic susceptibility testing was performed with the broth dilution method according to CLSI guidelines (M7-A7) (24). The following antimicrobial agents were used for antibiotic susceptibility testing: piperacillin, cefotaxime, cefepime, imipenem (all, Sigma Chemical, St. Louis, MO, USA); ciprofloxacin (LKT Laboratories, MN, USA); aztreonam (Tokyo Chemical Industry Co. Ltd., Tokyo, Japan); tazobactam (Toyama Chemical Co., Ltd., Toyama, Japan); moxalactam (Shionogi & Co., Ltd., Osaka, Japan); amikacin (Wako Pure Chemical Industries, Ltd., Tokyo, Japan). *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as the quality control strains for antibiotic susceptibility testing. The results were interpreted according to CLSI guidelines (36).

Acquired antimicrobial resistance genes were identified using the ResFinder, version 2.1, database (https://cge.cbs.dtu.dk/services/ResFinder/).

Plasmid analysis. Plasmid incompatibility replicon typing was performed using PlasmidFinder. Conjugation experiments of *bla*$_{\text{IMP-1}}$-carrying plasmids were performed with filter mating methods using a sodium azide-resistant mutant of the non-lactose-fermenting *E. coli* ML4909 strain (a rifampin-resistant mutant of *E. coli* K-12) as a recipient. Transconjugants were selected on Drigalski lactose agar supplied with moxalactam (16 μg/ml) and sodium azide (150 μg/ml). Carriage of *bla*$_{\text{IMP-1}}$ by transconjugants was confirmed by PCR, and the Inc type of *bla*$_{\text{IMP-1}}$-carrying plasmids was determined by PCR-based Inc/Rep typing (37). Conjugation was repeated up to three times if initial attempts were unsuccessful.

Complete nucleotide sequences of representative plasmids of IncW and IncFIB (pMTY10660_IncW and pMTY10695_IncFIB, respectively) were determined manually by connecting the edges of relevant contigs with PCR and Sanger sequencing. To obtain the complete sequence of a representative IncHI2 plasmid (pMTY11043_IncHI2), we used the PacBio RS system (Pacific Biosciences, Menlo Park, CA). The library was prepared using DNA Template Prep kit, version 1.0, and DNA/Polymerase Binding Kit P5 and then bound to MagBeads according to the manufacturer's instructions. Sequencing was performed with DNA Sequencing Kit C3 (Pacific Biosciences) by taking one 180-min movie for a single cell using SMRT (single-molecule, real-time) analysis. PacBio sequence reads were assembled *de novo* using a hierarchical genome assembly process (HGAP, version 3.0) in SMRT Pipe, version 1.85. We used the Circlator tool for automated circularization of plasmid genome assemblies using PacBio long sequencing reads (38). The annotation of the plasmid sequences was conducted by DFAST (39) and edited manually. Integrons were classified according to the Integrall database (http://integrall.bio.ua.pt/). *In silico* plasmid multilocus

sequence typing of conjugative IncHI2 plasmids carrying bla_{IMP-1} was performed with whole-genome sequencing data (40). Comparison of conjugative IncHI2 plasmids carrying bla_{IMP-1} and IncFIB plasmids of clade 3 isolates was performed with BLAST Ring Image Generator (BRIG), version 0.95 (41). Comparison of the sequences of representative plasmids of each Inc group in this study with those of similar plasmids reported previously was performed and visualized with EasyFig, version 2.1 (42).

Accession number(s). The results of this study were deposited in the NCBI database under BioProject number PRJDB6127. The draft genome sequences of 71 ECC isolates were deposited in DDBJ and the NCBI BioSample database under accession numbers SAMD00089454 to SAMD00089524.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.02091-17.

SUPPLEMENTAL FILE 1, PDF file, 2.1 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.1 MB.

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