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Wastewater as a Probable Environmental Reservoir of Extended-Spectrum-β-Lactamase Genes: Detection of Chimeric β-Lactamases CTX-M-64 and CTX-M-123

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ABSTRACT The presence of antimicrobial-resistant bacteria and resistance genes in aquatic environments is a serious public health concern. This study focused on *Escherichia coli* possessing bla_{CTX-M} genes in wastewater inflows. Twelve crude inflow water samples from wastewater treatment plant (WWTP) A and two samples each from three other WWTPs were collected in 2017 and 2018. A total of 73 *E. coli* isolates with 31 different sequence types (STs) harboring distinctive bla_{CTX-M} gene repertoires were detected. In WWTP A influents, $bla_{CTX-M-14}$ (14 isolates) was dominant, followed by $bla_{CTX-M-15}$ (12 isolates) and $bla_{CTX-M-27}$ (10 isolates). The chimeric $bla_{CTX-M-64}$ and $bla_{CTX-M-123}$ genes were each identified in one of the *E. coli* isolates from the same WWTP A inflow port. The $bla_{CTX-M-27}$ gene was associated with five of seven B2-ST131 isolates, including three isolates of the B2-O25b-ST131-*H*30R/non-Rx lineage. One of the remaining two isolates belonged to the B2-O25b-ST131-*H*30R/Rx lineage harboring the $bla_{CTX-M-15}$ gene. As for the B2-O25b-ST131-*H*30R/non-Rx lineage, two isolates with $bla_{CTX-M-27}$ were recovered from each of the WWTP B and D influents, and one isolate with $bla_{CTX-M-174}$ was also recovered from WWTP B influent. Whole-genome sequencing of chimeric bla_{CTX-M}-harboring *E. coli* isolates revealed that the $bla_{CTX-M-64}$ gene was integrated into the chromosome of ST10 *E. coli* B22 via IS*Ecp1*-mediated transposition of a 9,467-bp sequence. The $bla_{CTX-M-123}$-carrying IncI1 plasmid pB64 was 109,169 bp in length with pST108. The overall findings suggest that wastewater may act as a probable reservoir of clinically significant clonal lineages mediating antimicrobial resistance genes and chimeric genes that have not yet been identified from human isolates of domestic origin in Japan.

IMPORTANCE Global spread of CTX-M-type extended-spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae* is a critical concern in both clinical and community settings. This dominance of CTX-M-type ESBL producers may be largely due to the successful international spread of epidemic clones, as represented by the extraintestinal pathogenic *Escherichia coli* (ExPEC) ST131. Our findings highlight the worrisome presence of diverse *E. coli* clones associated with humans, including ExPEC lineages harboring the most common bla_{CTX-M} variants in untreated wastewater samples. Moreover, the chimeric genes $bla_{CTX-M-64}$ and $bla_{CTX-M-123}$, which have not yet been identified from human isolates of domestic origin in Japan, were identified. Exposure to untreated wastewater through combined sewer overflow caused by heavy rains derived from abnormal weather change could pose a risk for human health due to ingesting those antimicrobial-resistant bacteria.

KEYWORDS wastewater, ESBL, CTX-M-64, CTX-M-123, WWTPs

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The worldwide spread of extended-spectrum β-lactamase (ESBL)-producing bacteria, particularly *Escherichia coli* and *Klebsiella pneumoniae*, emerging as multidrug-resistant microbes in both clinical and community settings is a critical concern. The variations in the intestinal carriage of ESBL producers in healthy individuals have been observed among countries, especially in Africa and Asia (15 to 46%), including Japan (3 to 16%), compared to Europe (3 to 6%) and the Americas (2%) (1, 2). Asymptomatic intestinal carriage of ESBL producers could be linked to developing urinary tract and more severe invasive infections, contributing to the transmission of ESBL producers within the community as their reservoirs, as well as to the potential influx of ESBL producers into hospital settings from the community (3).

CTX-M type β-lactamases, which are the representative ESBLs, have been recognized as the most common community-derived ESBLs during the past decade (1). More than 220 different CTX-M enzymes have been deposited in GenBank to date. CTX-M-type ESBLs have been classified roughly into four groups based on their amino acid sequence similarities: CTX-M-1, CTX-M-2, CTX-M-9, and CTX-M-8/CTX-M-25. Among the CTX-M-type ESBLs exhibiting rapidly growing diversity in genotypes, the most predominant variants currently are CTX-M-15, which belongs to the CTX-M-1 group found in many countries across the world, including the United Kingdom, the United States, Canada, Africa, and India, and CTX-M-14, which belongs to the CTX-M-9 group reported especially in Asian countries, including Japan and China, as well as Spain (4–6). A high prevalence of CTX-M-1 has also been described in Italy and France. Notably, the recent rise of CTX-M-27, an Asp240Gly variant of CTX-M-14, exhibiting a higher ceftazidime MIC compared to CTX-M-14, has been documented globally (4, 5). The global dominance of CTX-M-15, CTX-M-14, and CTX-M-27 producers may be largely due to the successful international spread of epidemic clones, as represented by the extraintestinal pathogenic *E. coli* (ExPEC) ST131 clones belonging to phylogenetic group B2 with the specific serotype O25b:H4. This B2-O25b:H4-ST131 lineage harbors transferable plasmids mediating CTX-M-type ESBL genes, as well as other antimicrobial resistance genes, allowing their efficient dissemination among the family *Enterobacteriaceae* (4, 5).

There is growing concern that *E. coli* isolates producing CTX-M enzymes, particularly CTX-M-15 and CTX-M-14, have also been frequently detected in companion animals, food-producing animals and their meat, and environmental samples in many countries (7–11). Furthermore, a high prevalence of CTX-M-27 producers among companion animals and a relatively high incidence of CTX-M-55, an Ala77Val variant from CTX-M-15 among raw chicken meat in Japan, have been documented in our own recent studies (10, 11). The importance of the role of environmental sources in spreading ESBL producers into the community has been investigated (4); however, little is known regarding the occurrence of ESBL producers in aquatic environments in Japan.

We sought to investigate the resistance characteristics and genetic backgrounds of $bla_{\text{CTX-M}}$-carrying *E. coli* recovered from untreated wastewater. Particularly noteworthy was the detection of $bla_{\text{CTX-M-64}}$ and $bla_{\text{CTX-M-123}}$ genes, encoding CTX-M-64 and CTX-M-123, respectively, chimeric genes of $bla_{\text{CTX-M-15}}$ and $bla_{\text{CTX-M-14}}$, despite the fact that there have been no reports so far of such chimeric genes from human isolates of domestic origin in Japan (12, 13).

RESULTS

***E. coli* clonal lineages and $bla_{\text{CTX-M}}$ gene repertoires detected from influents of municipal wastewater treatment plants (WWTPs) A, B, C, and D.** A total of 73 *E. coli* isolates comprising 31 different clonal lineages harboring distinct $bla_{\text{CTX-M}}$ gene repertoires were detected from WWTPs A, B, C, and D. Table 1 shows the genotypic characteristics of 50 *E. coli* isolates recovered from three inflow ports (A-1, A-2, and A-3) of WWTP A over 4 months. The dominant $bla_{\text{CTX-M}}$ genes found were $bla_{\text{CTX-M-14}}$ (14 isolates), $bla_{\text{CTX-M-15}}$ (12 isolates), and $bla_{\text{CTX-M-27}}$ (10 isolates), which were followed by $bla_{\text{CTX-M-55}}$ (5 isolates), $bla_{\text{CTX-M-3}}$ (3 isolates), $bla_{\text{CTX-M-1}}$ (1 isolate), $bla_{\text{CTX-M-2}}$ (1 isolate), $bla_{\text{CTX-M-24}}$ (1 isolate), and $bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-15}}$ (1 isolate). Of note, the chimeric genes $bla_{\text{CTX-M-64}}$ and $bla_{\text{CTX-M-123}}$ were each identified in one of the *E. coli* isolates from

TABLE 1 Genotypic characteristics of CTX-M-producing *E. coli* isolates recovered from WWTP A influents

			Inflow port A-1 (n = 20)				Inflow port A-2 (n = 15)				Inflow port A-3 (n = 15)			
Date, no. of isolates (n = 50)	bla_{CTX-M} gene	n	Phylogroup	ST	Serotype O25b	Subclone/ lineage	Phylogroup	ST	Serotype O25b	Subclone/ lineage	Phylogroup	ST	Serotype O25b	Subclone/ lineage
September 2017, 11	$bla_{CTX-M-27}$	3	B2	ST2252[a]	Non-O25b	Non-H30	B2	ST131	Non-O25b	Non-H30				
			F	ST648										
	$bla_{CTX-M-55}$	2	A	ST10[a,b]			F	ST648						
	$bla_{CTX-M-14}$	2					F	ST648[c]			C	ST167[c]		
	$bla_{CTX-M-15}$	1					B2	ST127	Non-O25b	Non-H30				
	$bla_{CTX-M-3}$	1	B1	ST4720										
	$bla_{CTX-M-1}$	1	B1	ST2073-like[a]										
	$bla_{CTX-M-64}$	1					C	ST10						
October 2017, 15	$bla_{CTX-M-14}$	5	D	ST38			D	ST38						
			F	ST354[c]			F	ST354[c]						
			F	ST648										
	$bla_{CTX-M-15}$	3	A	ST10							A	ST44[c]		
											D	ST405		
	$bla_{CTX-M-3}$	2	B1	ST156[a,c]										
			B2	ST127	Non-O25b	Non-H30								
	$bla_{CTX-M-55}$	2					B1	ST155			D	ST5150		
	$bla_{CTX-M-27}$	2					B2	ST131	Non-O25b	Non-H30	F	ST1722		
	$bla_{CTX-M-2}$	1									F	ST117		
November 2017, 12	$bla_{CTX-M-14}$	5	D	ST38			F	ST393[c]			A	ST10[c]		
											B2	ST131[a]	Non-O25b	Non-H30
											F	ST10[c]		
	$bla_{CTX-M-15}$	4	A	ST43			B1	ST4681						
			A	ST617										
			F	ST648										
	$bla_{CTX-M-24}$	1									F	ST354[c]		
	$bla_{CTX-M-27}$	1	B2	ST131	O25b	*H*30R/non-Rx								
	$bla_{CTX-M-15}$ and $bla_{CTX-M-14}$	1					F	ST648[a]						
December 2017, 12	$bla_{CTX-M-15}$	4	A	ST10			A	ST43			A	ST43		
											B2	ST131[a]	O25b	*H*30R/Rx
	$bla_{CTX-M-27}$	4	A	ST46[a]			B2	ST131[a]	O25b	*H*30R/non-Rx	D	ST69[a]		
			B2	ST131[b]	O25b	*H*30R/non-Rx								
	$bla_{CTX-M-14}$	2	D	ST69[c]							D	ST38		
	$bla_{CTX-M-55}$	1									B2	ST676	O25b	Non-H30
	$bla_{CTX-M-123}$	1					F	ST1674[d]						

[a]Positive for *tetA* gene.
[b]Positive for *fosA3* gene.
[c]Positive for *tetB* gene.
[d]The isolate also carried the bla_{CMY-2} gene.

inflow port A-2 at 3-month intervals. The 50 *E. coli* isolates were assigned to 25 distinct STs, where seven isolates were found to be ST131 clone belonging to phylogroup B2. The $bla_{CTX\text{-}M\text{-}27}$ gene was associated with five of these seven isolates of the clone B2-ST131, including three isolates of the B2-O25b-ST131-*H*30R/non-Rx lineage. As for the remaining two isolates of the clone B2-ST131, one isolate harboring $bla_{CTX\text{-}M\text{-}15}$ was identified to be of the B2-O25b-ST131-*H*30R/Rx lineage. Six isolates belonged to one secondary dominant ST648 clone with phylogroup F though they harbored different $bla_{CTX\text{-}M}$ genes, $bla_{CTX\text{-}M\text{-}14}$ (two isolates), and $bla_{CTX\text{-}M\text{-}15}$, $bla_{CTX\text{-}M\text{-}27}$, $bla_{CTX\text{-}M\text{-}55}$, and $bla_{CTX\text{-}M\text{-}14/15}$ (one isolate each). Six isolates of another secondary dominant ST10 clone belonged to three different phylogroups, including phylogroups A (two isolates, one isolate, and one isolate harboring $bla_{CTX\text{-}M\text{-}15}$, $bla_{CTX\text{-}M\text{-}55}$, and $bla_{CTX\text{-}M\text{-}14}$, respectively), C (one isolate harboring $bla_{CTX\text{-}M\text{-}64}$), and F (one isolate harboring $bla_{CTX\text{-}M\text{-}14}$). All four isolates of the ST38 clone belonging to phylogroup D carried $bla_{CTX\text{-}M\text{-}14}$. Two isolates harboring $bla_{CTX\text{-}M\text{-}15}$ and $bla_{CTX\text{-}M\text{-}3}$ each were identified to be the phylogroup B2-ST127 clone (Table 1). *E. coli* B2-ST131, F-ST648, A-ST10, D-ST38, and B2-ST127 clones were detected across different sampling months, inflow ports at WWTP A, and different WWTPs.

Table 2 shows genotypic characteristics of 23 *E. coli* isolates recovered from influents of WWTPs B, C, and D. The B2-O25b-ST131-*H*30R/non-Rx lineage with $bla_{CTX\text{-}M\text{-}27}$ was identified in both samples from WWTPs B and D. Interestingly, despite belonging to the B2-O25b-ST131-*H*30R/non-Rx lineage, an isolate possessing $bla_{CTX\text{-}M\text{-}174}$ was recognized. The B2-non-O25b-ST131-non-*H*30 lineage isolates with $bla_{CTX\text{-}M\text{-}14}$ (two isolates) and $bla_{CTX\text{-}M\text{-}27}$ (two isolates) were found from influents of WWTPs B/C and WWTP D, respectively. Four isolates harboring $bla_{CTX\text{-}M\text{-}14}$ belonged to different sequence types (STs): ST38, ST58, ST69, and ST206.

Prevalence of other antimicrobial resistance genes. Among 73 *E. coli* isolates harboring $bla_{CTX\text{-}M}$ genes, the plasmid-mediated 16S rRNA methyltransferase genes *armA*, *rmtB*, and *rmtC* and the plasmid-mediated colistin resistance *mcr* genes *mcr-1*, *mcr-2*, and *mcr-3* were not detected. The frequency of the tetracycline resistance genes *tetA* and *tetB* and the plasmid-mediated fosfomycin resistance gene *fosA3* were as follows: 8 isolates were positive for *tetA*, 10 were positive for *tetB*, 1 was positive for *fosA3*, 1 was positive for *tetA* and *tetB*, and 1 was positive for *tetA* and *fosA3* in WWTP A influents (Table 1); 9 isolates were positive for *tetA*, 1 was positive for *tetB*, and 2 were positive for *tetA* and *fosA3* in WWTP B, C, and D influents (Table 2).

Susceptibility testing of chimeric $bla_{CTX\text{-}M}$ gene-harboring *E. coli* isolates. The MICs of various antimicrobials for *E. coli* B22 harboring $bla_{CTX\text{-}M\text{-}64}$, *E. coli* B64 harboring plasmid pB64 with $bla_{CTX\text{-}M\text{-}123}$, and *E. coli* χ1037 transconjugant that acquired plasmid pB64 with $bla_{CTX\text{-}M\text{-}123}$ are listed in Table 3. *E. coli* B22 did not yield cefotaxime (CTX)-resistant transconjugant or electrotransformant in multiple experiments. *E. coli* B22, *E. coli* B64, and *E. coli* χ1037 transconjugant that acquired plasmid pB64 were resistant to all the third-generation cephalosporins and to aztreonam, whereas they were susceptible to carbapenems. *E. coli* B22 had a cefotaxime MIC of >2,048 mg/liter and a ceftazidime MIC of 64 mg/liter. *E. coli* B64 and the *E. coli* χ1037 transconjugant that acquired plasmid pB64 had cefotaxime MICs of 2,048 mg/liter and ceftazidime MICs of 32 mg/liter. Consistent results for susceptibility profiles, including a cefotaxime MIC of >2,048 mg/liter and a ceftazidime MIC of 64 mg/liter, were observed for the *E. coli* χ1037 transconjugant harboring pUIH-1. Levofloxacin resistance in *E. coli* B22 was not observed in *E. coli* B64 and two *E. coli* χ1037 transconjugants.

Genetic features of $bla_{CTX\text{-}M\text{-}64}$ gene-harboring *E. coli* B22. Genome assembly of *E. coli* B22 yielded 273 contig sequences with the total assembly length of 5,084,128 bp, GC content of 50.6%, and an N_{50} of 131,142 bp. Gene annotation identified 4,670 protein-coding genes, 87 tRNA genes, and 7 rRNA genes. *E. coli* B22 belonged to the ST10 clone, O8:H17 serotype, and *fimH41* subclone and contained the $bla_{CTX\text{-}M\text{-}64}$ gene and substitutions conferring quinolone resistance, Ser80Ile in ParC and Ser83Leu/Asp87Asn in GyrA (Table 4). The virulence genes *astA*, *cma*, and *gad* were also detected.

TABLE 2 Genotypic characteristics of CTX-M-producing *E. coli* isolates recovered from WWTP B, C, and D influents

WWTP (mo yr)	No. of isolates ($n = 23$)	bla_{CTX-M} gene	No. of specimens	Finding in the indicated location							
				Phylogroup	ST	Serotype O25b	Subclone/ lineage	Phylogroup	ST	Serotype O25b	Subclone/ lineage
B (July 2018)	7			Inflow port B-1 ($n = 5$)				Sampling point[a] B-2 ($n = 2$)			
		$bla_{CTX-M-55}$	2	B1	58[b]						
				E clade	10[b,c]						
		$bla_{CTX-M-14}$	1	B2	131[b]	Non-O25b	Non-H30				
		$bla_{CTX-M-15}$	1					D	70[e]		
		$bla_{CTX-M-27}$	2	B2	131	O25b	*H*30R/non-Rx	B2	131[b]	O25b	*H*30R/non-Rx
		$bla_{CTX-M-174}$	1	B2	131[b]	O25b	*H*30R/non-Rx				
C (August 2018)	4			Inflow port C-1 ($n = 2$)				Sampling point[a] C-2 ($n = 2$)			
		$bla_{CTX-M-14}$	2					B2	131	Non-O25b	Non-H30
								D	38		
		$bla_{CTX-M-15}$	1	F	648						
		$bla_{CTX-M-65}$	1	B1	2179						
D (September 2018)	12			Inflow port D-1 ($n = 6$)				Inflow port D-2 ($n = 6$)			
		$bla_{CTX-M-27}$	5	B2	131[b]	Non-O25b	Non-H30	B2	131	Non-O25b	Non-H30
				B2	131[b]	O25b	*H*30R/non-Rx	B2	131	O25b	*H*30R/non-Rx
								D	2003		
		$bla_{CTX-M-14}$	4	D	69[d]			D	38		
								B1	58[b]		
								A	206[b]		
		$bla_{CTX-M-8}$	1	B1	345						
		$bla_{CTX-M-15}$	1	D	69[b]						
		$bla_{CTX-M-55}$	1	B1	767[b,c]						

[a]The sample contained inflow water and return flows from sludge treatment.
[b]Positive for *tetA* gene.
[c]Positive for *fosA3* gene.
[d]Positive for *tetB* gene.
[e]The isolate also carried the bla_{DHA-1} gene.

TABLE 3 MICs of antimicrobials for the CTX-M-64-producing *E. coli* strain B22 and CTX-M-123-producing *E. coli* B64 strain and its transconjugants

Antimicrobial	MIC (mg/liter)				
	B22 ($bla_{CTX-M-64}$)	B64 ($bla_{CTX-M-123}$)	χ1037 Rif^r transconjugant ($bla_{CTX-M-123}$/pB64)	χ1037 Rif^r transconjugant[b] ($bla_{CTX-M-64}$/pUIH-1)	χ1037 Rif^r
Piperacillin	>64	>64	>64	>64	≤0.25
SAM[c]	8/16	8/16	8/16	8/16	≤2/4
Cefazolin	>16	>16	>16	>16	1
Cefotiam	>16	>16	>16	>16	≤0.25
Cefotaxime[a]	>2,048	2,048	2,048	>2,048	≤0.25
Ceftazidime[a]	64	32	32	64	≤0.25
Cefpodoxime	>4	>4	>4	>4	≤1
Cefepime	8	>16	>16	>16	≤0.25
Flomoxef	≤0.5	16	≤0.5	≤0.5	≤0.5
Aztreonam	>16	>16	16	>16	≤0.5
Imipenem	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25
Meropenem	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25
Gentamicin	≤0.25	0.5	≤0.25	1	≤0.25
Amikacin	4	4	≤1	2	≤1
Minocycline	4	0.5	1	1	1
Levofloxacin	>4	≤0.25	≤0.25	≤0.25	≤0.25
Fosfomycin	≤32	≤32	≤32	≤32	≤32
SXT[c]	≤9.5/0.5	≤9.5/0.5	≤9.5/0.5	≤9.5/0.5	≤9.5/0.5

[a]The antibiotic-containing plates were prepared in-house.
[b]As described by Nagano et al. (12).
[c]SAM, ampicillin-sulbactam; SXT, trimethoprim-sulfamethoxazole.

Next-generation sequencing (NGS) data of $bla_{CTX-M-64}$-carrying plasmid UIH-1 generated 14 assembled contigs, which were subjected to gap-closing PCR and Sanger sequencing to determine the complete nucleotide sequence. The IncI2 plasmid UIH-1 was 62,194 bp, with a GC content of 42.1% and harboring 73 protein-coding genes.

Comparison of the genetic structure of the region surrounding the $bla_{CTX-M-64}$ gene revealed that a 9,467-bp sequence of IS*Ecp1*-$bla_{CTX-M-64}$-*orf477Δ-yajA-yaiB-orf1-dnaJ-orf2-yajB-parA-orf3-yaeC-flmC-orf4-orf5-orf6* in pUIH-1 shared 99.99% identity with the 9,467-bp corresponding region of *E. coli* B22 (Fig. 1A). It is noteworthy that at least a 157-kb region of the *E. coli* B22 genomic sequence, with the exception of the above 9,467-bp region, exhibited a high degree of sequence identity (99.99%) with the 147.6-kb chromosomal sequence of *E. coli* Y5 (GenBank accession no. CP013483). The 9,467-bp region was flanked by 5-bp direct repeats (TATAA), which were integrated between open reading frames (ORFs), ManX (PTS system mannose-specific EIIAB component), and YoaE (inner membrane protein) of the *E. coli* B22 genomic sequence (Fig. 1A).

Moreover, a 14,193-bp sequence immediately upstream region of the IS*Ecp1*-$bla_{CTX-M-64}$ in pUIH-1 shared 99.98% sequence identity with the 14,193-bp corresponding region of *E. coli* B22 (Fig. 1A). The 14,193-bp region was flanked by 5-bp direct repeats (TAAAT), which were integrated between ORFs MprA (negative regulator of the

TABLE 4 WGS-based analysis of *E. coli* B22 harboring $bla_{CTX-M-64}$ and *E. coli* B64 harboring $bla_{CTX-M-123}$

Genetic characteristics		*E. coli* B22	*E. coli* B64
Antimicrobial resistance genes	Chromosome(s)	$bla_{CTX-M-64}$, *mdfA* Ser80Ile in ParC, Ser83Leu and Asp87Asn in GyrA	bla_{CMY-2}, *mdfA*
	Plasmid(s)	bla_{TEM-1B}	$bla_{CTX-M-123}$, *mphA*
Virulence genes	Chromosome	*astA*, *gad*	*air*, *eilA*, *lpfA*
	Plasmid	*cma*	
Plasmid replicon type(s)		IncFIB, IncFII, IncI1	IncI1
MLST		ST10	ST1674
Serotype		O8:H17	O11:H25
fimH type		*fimH41*	*fimH138*

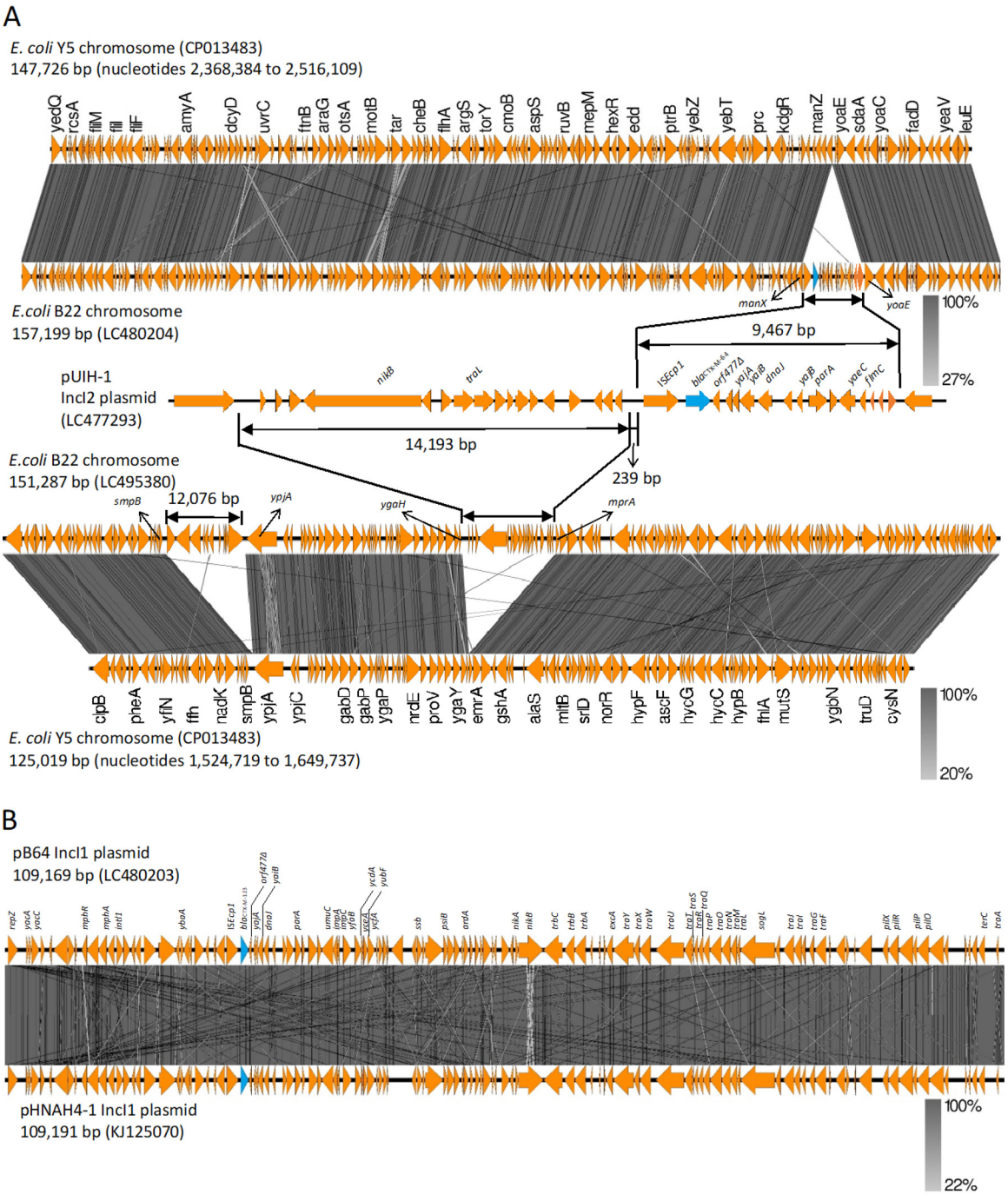

FIG 1 Schematic representation of the chimeric genes. (A) Genome alignments of *E. coli* B22, including the 9,467-bp sequence of IS*Ecp1-bla*$_{CTX-M-64}$*-orf477Δ-yajA-yaiB-orf1-dnaJ-orf2-yajB-parA-orf3-yaeC-flmC-orf4-orf5-orf6* (GenBank accession number LC480204) with *E. coli* Y5 (CP013483), and of *E. coli* B22, including 14,193-bp sequence immediately upstream region of the IS*Ecp1-bla*$_{CTX-M-64}$ (GenBank accession number LC495380) with *E. coli* Y5 (CP013483), and comparison of genetic environment flanking *bla*$_{CTX-M-64}$ in *E. coli* B22 to that of plasmid pUHI from *S. sonnei* (GenBank accession number LC477293). (B) Sequence comparison between plasmid pB64 carrying *bla*$_{CTX-M-123}$ (GenBank accession number LC480203) and pHNAH4-1 (KJ125070). The arrows show the translation orientation of the coding genes. Figure was generated using EasyFig (http://mjsull.github.io/Easyfig/).

multidrug operon *emrAB*) and YgaH (uncharacterized protein) of the *E. coli* B22 genomic sequence (Fig. 1A).

Genetic features of the *bla*$_{CTX-M-123}$ gene-harboring *E. coli* B64. The WGS assembly of *E. coli* B64 contained 316 contigs, with a total length of 5,042,491 bp, a GC content of 50.3%, and an N_{50} of 199,993 bp. Gene annotation identified 4,544 protein-

coding genes, 82 tRNA genes, and 8 rRNA genes. *E. coli* B64 belonged to the ST1674 clone, O11:H25 serotype, and *fimH138* subclone and contained $bla_{CTX\text{-}M\text{-}123}$ and $bla_{CMY\text{-}2}$ resistance genes and *air*, *eilA*, *lpfA* virulence genes (Table 4).

NGS-based plasmid sequencing of $bla_{CTX\text{-}M\text{-}123}$-carrying plasmid pB64 yielded 57 assembled contigs, which were subjected to gap-closing PCR and Sanger sequencing to determine the complete nucleotide sequence. The IncI1 plasmid pB64 with ST108 by pMLST was 109,169 bp, with a GC content of 50.6% harboring 124 protein-coding genes. The plasmid pB64 shared 99.97% sequence identity with that of pHNAH4-1 (IncI1 ST108, GenBank KJ125070) harbored by the *E. coli* ST746 isolate from chicken feces in China (14) (Fig. 1B).

The genetic context of $bla_{CMY\text{-}2}$ was investigated by exploring ~350-kbp fragments of the *E. coli* B64 genomic sequence. The results revealed that a 10,178-bp sequence, including IS*Ecp1*-*bla*CMY-2-Δ*blc*-*yggR*-Δ*tnp1*-*orf7*-*orf8*-*orf9*-Δ*tnp2*-Δ*hsdR*, sharing 100% identity with the 10,178-bp corresponding region of the *E. coli* CRE10 isolate from a human stool sample in Thailand (CP034404), was located on its chromosome. The macrolide resistance operon *mphA*-*mrx*-*mphR* located between two IS*26* elements was associated with class 1 integrase gene *intI1*, which carried no class 1 integron gene cassettes.

DISCUSSION

Exposure to untreated wastewater could pose a risk for human health due to the ingestion of unrecognized antimicrobial-resistant bacteria. Particularly, the combined sewer overflow that has frequently occurred in recent years caused by localized heavy rains resulting from abnormal weather changes may diffuse those resistant organisms into the living environment. Thus, in the present study, the untreated wastewater samples flowing into inflow ports of WWTPs were investigated in order to gain better understanding into their probable risks for human health via exposure to clinically significant clonal lineages of ESBL-producing *E. coli*.

Despite the high overall genetic diversity of 31 STs among the 73 $bla_{CTX\text{-}M}$-harboring *E. coli* isolates detected in untreated wastewater flows, clinically important lineages ST131 (16 isolates), ST10 (7 isolates), ST648 (7 isolates), ST38 (6 isolates), and ST69 (4 isolates) were noted at higher frequencies. The highly successful epidemic clone ST131, a major contributor to ExPEC infections in humans, has been broadly disseminated among nonhuman sources, such as companion animals, food animals, foods of animal origin, and environmental samples (15). Particularly, the global spread of the specific epidemic subclone B2-O25b-ST131-*H*30R mediating $bla_{CTX\text{-}M\text{-}15}$, $bla_{CTX\text{-}M\text{-}27}$, or $bla_{CTX\text{-}M\text{-}14}$ poses a major public health threat because of its multidrug-resistant and potential virulent characteristics (16, 17). Recently, we have described the high prevalence of the $bla_{CTX\text{-}M\text{-}27}$ gene among the pandemic B2-O25b-ST131-*H*30R/non-Rx lineage isolates from companion animals in Japan (10). The present study shows that this B2-O25b-ST131-*H*30R/non-Rx *E. coli* carrying $bla_{CTX\text{-}M\text{-}27}$ was detected from influents of all WWTPs except those of the WWTP C, in addition to the B2-O25b-ST131-*H*30R/Rx *E. coli* carrying $bla_{CTX\text{-}M\text{-}15}$ from WWTP A influent. Interestingly, $bla_{CTX\text{-}M\text{-}174}$, a Glu7Leu variant of $bla_{CTX\text{-}M\text{-}27}$ (only nucleotide sequence data are available in the GenBank database under the accession number KT997886), was also associated with the B2-O25b-ST131-*H*30R/non-Rx lineage, raising concerns regarding the future spread of this lineage carrying $bla_{CTX\text{-}M\text{-}174}$ in both hospital and community settings. Moreover, in the present study, the $bla_{CTX\text{-}M\text{-}27}$ was detected among several other lineages, such as B2-non-O25b-ST131-non-*H*30, B2-non-O25b-ST2252-non-*H*30, A-ST46, D-ST69, D-ST2003, F-ST648, and F-ST1722, although Matsumura et al. have reported that the $bla_{CTX\text{-}M\text{-}27}$ was confined to the O25b-ST131-*H*30R/non-Rx lineage of human clinical isolates (17). Phylogroup D constitutes the second major ExPEC lineage after phylogroup B2. *E. coli* clones ST38, ST69, ST405, ST70, ST2003, and ST5150 were recognized among this phylogroup D, with ST38, which has been recognized to carry virulence determinants of both uropathogenic *E. coli* and enteroaggregative *E. coli* (18), being the most common (6 of 14 isolates).

Clinically relevant *E. coli* lineages, including globally circulating B2-ST131, F-ST648, A-ST10, D-ST38, and the recently emerged virulent clone B2-ST127, were detected across different sampling months and inflow ports of the WWTP A or across different WWTPs, suggesting these lineages have acquired the ability to adapt to the environment; this highlights the importance of untreated wastewater flow as an unrecognized crucial reservoir of these epidemic lineages.

The present study confirmed the overall predominance of phylogroups B2 and D among the $bla_{CTX\text{-}M}$-harboring *E. coli* isolates derived from untreated domestic wastewater flows. Similar findings have been reported previously among ESBL-producing *E. coli* isolates from human commensal populations, as well as from WWTP effluents (19, 20). Nonetheless, this study newly identifies the $bla_{CTX\text{-}M\text{-}174}$ gene associated with the B2-O25b-ST131-*H*30R/non-Rx lineage and the uncommon chimeric genes $bla_{CTX\text{-}M\text{-}64}$ and $bla_{CTX\text{-}M\text{-}123}$, which probably originated by recombination between $bla_{CTX\text{-}M\text{-}14}$ and $bla_{CTX\text{-}M\text{-}15}$. The $bla_{CTX\text{-}M\text{-}64}$ was first identified by Nagano et al. as a chimeric ESBL gene in a *Shigella sonnei* strain UIH-1 recovered from a tourist who had returned from China (12). Subsequently, several chimeric $bla_{CTX\text{-}M}$ genes, including $bla_{CTX\text{-}M\text{-}123}$, have been reported from pets, food animals, and humans, mostly in China (13, 21), and also recently in a German patient (22). The prevalence of the $bla_{CTX\text{-}M\text{-}64}$ gene has been determined to be 6% among ESBL-producing *E. coli* isolates recovered from fecal samples from healthy children in Laos (23). In Japan, the occurrence of chimeric $bla_{CTX\text{-}M}$ genes has been associated with a travel-associated *S. sonnei* isolate from China for $bla_{CTX\text{-}M\text{-}64}$ and with an *E. coli* isolate from a domestic pet dog for $bla_{CTX\text{-}M\text{-}123}$; no chimeric genes from human isolates of domestic origin have yet been reported (12, 24).

CTX-M-64 and CTX-M-123, showing chimeric structures of a CTX-M-15 β-lactamase (N- and C-terminal moieties) and a CTX-M-14 β-lactamase (central portion), had amino acid sequence identities of 91.8 and 94.5%, respectively, with CTX-M-15, and of 88.0 and 85.2%, respectively, with CTX-M-14. The cefotaxime MICs for *E. coli* B22 producing CTX-M-64 and *E. coli* B64 producing CTX-M-123 were $>$2,048 mg/liter and 2,048 mg/liter, respectively, higher than those for *E. coli* transformants producing CTX-M-15 or CTX-M-14 reported previously (25). Also, it has been reported that these chimeric enzymes exhibited higher catalytic activities toward cefotaxime compared to CTX-M-15 and CTX-M-14, which was consistent with the MIC results (25).

The $bla_{CTX\text{-}M\text{-}64}$ gene was integrated into the chromosome of ST10 *E. coli* B22 via IS*Ecp1*-mediated transposition of a DNA fragment of 9,467-bp nucleotide sequence which showed 99.99% sequence identity to the corresponding sequence located on *S. sonnei* plasmid UIH-1. The distribution and high prevalence of chromosomally located $bla_{CTX\text{-}M}$ genes have been documented in Japan (26, 27). The stability of $bla_{CTX\text{-}M}$ genes integrated into the chromosome remains unclear, however, compared to the plasmid location; more stable retention of the chromosomally located antimicrobial resistance genes, allowing these organisms to live even in the absence of antimicrobial selection pressure, has been suggested. The occurrence of the chromosomal integration of $bla_{CTX\text{-}M\text{-}64}$ among the clinically important ESBL lineage could pose public health concerns due to antimicrobial resistance spread from untreated wastewater through combined sewer overflow, resulting in contamination of the human environment. Intriguingly, a 14,193-bp nucleotide sequence immediately upstream region of the IS*Ecp1*-$bla_{CTX\text{-}M\text{-}64}$, exhibiting 99.98% sequence identity with the corresponding sequence located on plasmid UIH-1, was integrated into another site on the *E. coli* B22 chromosome. This study reports an integration of divided segments probably derived from a $bla_{CTX\text{-}M\text{-}64}$-carrying IncI2 plasmid into *E. coli* chromosome at different sites. The mechanisms underlying this integration are unclear. Chromosome integration of the regions upstream and downstream of IS*Ecp1*-$bla_{CTX\text{-}M\text{-}64}$ may have occurred, followed by IS*Ecp1*-mediated transposition of $bla_{CTX\text{-}M\text{-}64}$ and its downstream fragments into another site on the chromosome. It is also possible that an independent integration of these 14,193- and 9,467-bp fragments occurs at different sites without significant homology on the chromosome following illegitimate recombination, as described previously (28).

The $bla_{CTX\text{-}M\text{-}123}$ gene has mainly been reported among food animal and human isolates, including *Shigella flexneri* in China, followed by isolation only among pet animals in the United States and Japan (24, 29, 30). The present study reveals that the IncI1 plasmid pB64 mediating the $bla_{CTX\text{-}M\text{-}123}$ gene harbored by *E. coli* F-ST1674 shares 99.97% sequence identity with the $bla_{CTX\text{-}M\text{-}123}$-carrying IncI1 plasmid pHNAH4-1 (GenBank accession no. KJ125070) from a chicken *E. coli* isolate in China. *E. coli* ST1674 is extremely rare in published articles, but the EnteroBase database (http://enterobase.warwick.ac.uk) reports ST1674 isolates detected in domestic water sources. *E. coli* ST1674 with the narrow-host-range IncI1 plasmid, which enables the development of the virulence factor type IV pili that are essential for adhesion and invasion, is more likely to be well adapted to a water environment, which thus may contribute to the horizontal transfer of $bla_{CTX\text{-}M}$ genes.

In conclusion, we demonstrated here the occurrence and repeated detection of $bla_{CTX\text{-}M}$ genes harbored by clinically significant *E. coli* lineages, including ST131, which is responsible for the spread of $bla_{CTX\text{-}M\text{-}15}$ and $bla_{CTX\text{-}M\text{-}27}$ in wastewater flow. Our present findings also show the emergence of $bla_{CTX\text{-}M\text{-}174}$, a variant of $bla_{CTX\text{-}M\text{-}27}$ among the B2-O25b-ST131-*H*30R/non-Rx lineage and chimeric genes of $bla_{CTX\text{-}M\text{-}64}$ and $bla_{CTX\text{-}M\text{-}123}$ characterized by enhanced catalytic activity to cephalosporins, all of which have not yet been identified in human isolates of domestic origin in Japan. Thus, wastewater may retain clinically important clonal lineages with antimicrobial resistance genes and chimeric genes spreading into human communities, including health care settings.

MATERIALS AND METHODS

Collection of wastewater inflows. A total of 12 crude inflow water samples were collected from WWTP A serving 125,000 people in the central area of Matsumoto City, Nagano Prefecture, Japan during September and December 2017; one sample each was collected from three different inflow ports A-1, A-2, and A-3, respectively, once a month for 4 months. In order to investigate whether there were any variations among ESBL-producing *E. coli* lineages or ESBL genes in wastewater samples depending on time and temperature, sampling on four occasions was performed in WWTP A. In addition, two samples each collected from B-1 (inflow water) and B-2 (inflow water plus return flows from sludge treatment) at WWTP B serving 162,000 people and C-1 (inflow water) and C-2 (inflow water plus return flows from sludge treatment) at WWTP C serving 134,000 people in Nagano City, and inflow ports D-1 and D-2 at WWTP D serving 95,000 people in Azumino City during July and September 2018 were included in our analyses.

Wastewater samples (~500 ml) collected in sterile glass bottles were transported to the laboratory under cooling conditions and were stored at 4°C until their utilization within 3 h after sampling.

Isolation of the cefotaxime-resistant *E. coli* and screening of ESBL production. Portions (1 ml) of well-mixed inflow samples were added to 9 ml of brilliant green lactose bile broth (BGLB broth; Eiken Chemical Co., Tokyo, Japan). After overnight incubation at 37°C, 10 μl of the broth was plated onto MacConkey agar (Eiken) supplemented with 2 mg/liter cefotaxime (CTX; Sigma-Aldrich Japan, Tokyo, Japan) and incubated overnight at 37°C. Several different colonies showing the expected morphological appearance of *E. coli* were subjected to matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Bruker Daltonics Japan, Yokohama, Japan) analysis. As recommended by the manufacturer, score cutoffs of ≥2.000 were applied for species-level identification.

Phenotypic screening of ESBL production on the basis of inhibitory effect of clavulanic acid was performed on Mueller-Hinton agar (Eiken) using CTX (30 μg; Eiken) and ceftazidime (30 μg; Eiken) disks, each alone and in combination with clavulanic acid (10 μg; Eiken) according to Clinical and Laboratory Standards Institute (CLSI) guidelines (31).

Genotype analysis of $bla_{CTX\text{-}M}$ genes. ESBL genes of $bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}2}$, $bla_{CTX\text{-}M\text{-}8}$, and $bla_{CTX\text{-}M\text{-}9}$ groups were detected by PCR with specific primers (11). The nucleotide sequences of the structural genes were determined by direct sequencing of PCR products by using BigDye Terminator v1.1 cycle sequencing kit and an ABI 3730xl genetic analyzer (Applied Biosystems, Foster City, CA) (11). The sequence results were analyzed using the NCBI BLAST program (https://www.ncbi.nlm.nih.gov/blast).

Molecular typing of *E. coli*. Phylotyping was performed using the Clermont multiplex PCR method with specific primers targeting *arpA*, *chuA*, *yjaA*, and TspE4.C2 (32).

Multilocus sequence typing (MLST) was carried out with seven housekeeping genes—*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA*—and the sequence type (ST) was assigned according to the *E. coli* MLST website (http://enterobase.warwick.ac.uk/species/ecoli/allele_st_search). If *E. coli* isolates sharing the same $bla_{CTX\text{-}M}$ genotypes, phylogroups, and STs were detected from the same samples, one representative isolate was included in the study data.

For *E. coli* isolates belonging to the phylogroup B2, the O25b molecular subtype was identified by PCR as described previously (33). Those isolates were further characterized; the *fimH*30 (*H*30) subclone was detected by PCR, and ciprofloxacin-resistant *H*30 isolates were identified as *H*30R (34), and the *H*30Rx

lineage within *H*30R subclone was identified by sequencing of PCR product for detection of the *H*30Rx-specific *ybbW* SNP G723A (35).

PCR detection of other resistance genes. For *E. coli* isolates harboring bla_{CTX-M} genes, the plasmid-mediated 16S rRNA methyltransferase genes *armA*, *rmtB*, and *rmtC* and the plasmid-mediated fosfomycin resistance gene *fosA3* were detected as described previously (10). Tetracycline resistance genes *tetA*, *tetB*, and *tetM* were detected by PCR with the following primers: *tetA*, 5′-GTAATTCTGAGCA CTGTCGC-3′ and 5′-TCAGCGATCGGCTCGT-3′; *tetB*, 5′-GTTCGACAAAGATCGCATTGG-3′ and 5′-AATCCAA ATCCAGCCATCCCA-3′; and *tetM*, 5′-TAGCTCATGTTGATGCAGGA-3′ and 5′-ATCCGACTATTTGGACGACGG-3′. The plasmid-mediated colistin resistance *mcr* genes *mcr-1*, *mcr-2*, and *mcr-3* were detected by PCR as described previously (36–38).

Analysis of chimeric $bla_{CTX-M-64}$- and $bla_{CTX-M-123}$-harboring *E. coli*. Whole-genome sequencing of $bla_{CTX-M-64}$-harboring *E. coli* B22 and $bla_{CTX-M-123}$-harboring *E. coli* B64, which were detected from influents at inflow port A-2 of WWTP A in September and December 2017, respectively, was performed. Genomic DNA was extracted by using a Wizard genomic DNA purification kit (Promega, Madison, WI). Sequencing libraries were prepared using the NEBNext Ultra DNA LibraryPrep kit (New England BioLabs, Ipswich, MA), and 150-bp paired-end sequencing was performed on the HiSeq platform (Illumina, San Diego, CA). *De novo* assembly was conducted using an A5-MiSeq Assembly pipeline with the default parameters (39). MLSTs, serotypes, *fimH* types, plasmid replicon types, antimicrobial resistance genes, and virulence genes were analyzed using tools available from the Center for Genomic Epidemiology (CGE; http://www.genomicepidemiology.org). Annotation of the resulting scaffolds was performed by using the DDBJ Fast Annotation and Submission Tool (DFAST; https://dfast.nig.ac.jp/) (40).

NGS-based plasmid sequencing of the plasmid pB64 carrying $bla_{CTX-M-123}$ from *E. coli* χ1037 transconjugants (donor *E. coli* B64) was performed. Plasmid pUIH-1 carrying $bla_{CTX-M-64}$ from *E. coli* χ1037 transconjugants obtained from the conjugation with *Shigella sonnei* strain UIH-1 was also analyzed for sequence comparison purposes (12). The plasmid DNA was extracted using the PureYield plasmid midiprep kit (Promega) and was subjected to pulsed-field gel electrophoresis. Plasmid DNA excised from the gel was used to prepare sequencing libraries using a Nextera XT DNA sample preparation kit (Illumina, San Diego, CA). Sequencing with 150-bp paired-end reads was performed on a MiSeq platform (Illumina). The contigs obtained after *de novo* assembly as described above were submitted to the DFAST for annotation. Plasmid MLSTs, plasmid replicon types, and antimicrobial resistance genes were analyzed using CGE web tools as described above.

Antimicrobial susceptibility testing of *E. coli* B22, *E. coli* B64, and *E. coli* χ1037 transconjugants that acquired plasmid pB64 carrying $bla_{CTX-M-123}$ was conducted by the broth microdilution method using Dry Plate Eiken (Eiken), and the results were interpreted by using the CLSI breakpoints (31). Alternatively, for cefotaxime and ceftazidime, broth microdilution panels prepared in-house were used to provide a broader range of antimicrobial concentrations for evaluation of the MICs. For comparative purposes, *E. coli* χ1037 transconjugant with plasmid pUIH-1 carrying $bla_{CTX-M-64}$ as described previously was also subjected to MIC determination (12).

Data availability. Two nucleotide sequences of *E. coli* B22 have been deposited in GenBank under accession numbers LC480204 (157,199 bp) and LC495380 (151,287 bp). The complete nucleotide sequences of pB64 and pUIH-1 have been deposited in the GenBank database under accession numbers LC480203 and LC477293, respectively.

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