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EPIDEMIOLOGY AND SURVEILLANCE

Characteristics of Carbapenemase-Producing *Enterobacteriaceae* in Wastewater Revealed by Genomic Analysis

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ABSTRACT Wastewater is considered a major source of antibiotic-resistant bacteria released into the environment. Here, we characterized carbapenemase-producing *Enterobacteriaceae* (CPE) in wastewater by whole-genome analysis. Wastewater samples ($n = 40$) were collected from municipal wastewater treatment plants and hospital wastewater in Japan and Taiwan. Samples were screened for CPE using selective media, and the obtained isolates were sequenced using an Illumina MiSeq. The isolates ($n = 45$) included the following microorganisms: *Klebsiella quasipneumoniae* ($n = 12$), *Escherichia coli* ($n = 10$), *Enterobacter cloacae* complex ($n = 10$), *Klebsiella pneumoniae* ($n = 8$), *Klebsiella variicola* ($n = 2$), *Raoultella ornithinolytica* ($n = 1$), *Citrobacter freundii* ($n = 1$), and *Citrobacter amalonaticus* ($n = 1$). Among the 45 isolates, 38 harbored at least one carbapenemase-encoding gene. Of these, the bla_{GES} (bla_{GES-5}, bla_{GES-6}, and bla_{GES-24}) genes were found in 29 isolates. The genes were situated in novel class 1 integrons, but the integron structures were different between the Japanese (In1439 with bla_{GES-24} and In1440 with bla_{GES-5}) and Taiwanese (In1441 with bla_{GES-5} and In1442 with bla_{GES-6}) isolates. Other carbapenemase-encoding genes (bla_{VIM-1}, bla_{NDM-5}, bla_{IMP-8}, bla_{IMP-19}, and bla_{KPC-2}) were found in one to three isolates. Notably, class 1 integrons previously reported among clinical isolates obtained in the same regions as the present study, namely, In477 with bla_{IMP-19} and In73 with bla_{IMP-8}, were found among the Japanese and Taiwanese isolates, respectively. The results indicate that CPE with various carbapenemase-encoding genes in different genetic contexts were present in biologically treated wastewater, highlighting the need to monitor for antibiotic resistance in wastewater.

KEYWORDS *Enterobacteriaceae*, carbapenemases, wastewater, whole-genome sequencing

Contamination of environmental waters by antibiotic-resistant bacteria (ARB) is a global health concern, because environmental waters are used for various purposes, including as sources of drinking water and for irrigation. Previous studies have reported fecal carriage of ARB in both community and clinical settings (1–4), indicating that wastewater released from municipal wastewater treatment plants (WWTPs) and hospitals can be a major source of environmental ARB.

Among ARB, carbapenemase-producing *Enterobacteriaceae* (CPE) are of great concern because these organisms can be resistant to carbapenems, which are often antimicrobial agents of last resort, and to other commonly used drugs (5). A large variety of carbapenemases have been reported, including those belonging to molecular Ambler class A, class B, and class D (6). Class A serine carbapenemases include members

Received 8 December 2017 **Returned for modification** 4 January 2018 **Accepted** 22 February 2018

Accepted manuscript posted online 26 February 2018

Citation Gomi R, Matsuda T, Yamamoto M, Chou P-H, Tanaka M, Ichiyama S, Yoneda M, Matsumura Y. 2018. Characteristics of carbapenemase-producing *Enterobacteriaceae* in wastewater revealed by genomic analysis. Antimicrob Agents Chemother 62:e02501-17. https://doi.org/10.1128/AAC.02501-17.

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of the SME, IMI, and GES enzymes, as well as clinically important KPC enzymes. Class B metallo-beta-lactamases include IMP, VIM, and NDM types of enzymes. Class D carbapenemases are OXA-type β-lactamases and include OXA-48 and OXA-181. Carbapenem resistance can also be conferred by mechanisms such as overexpression of β-lactamases possessing very weak carbapenemase activity in combination with decreased outer membrane permeability (7). However, resistance conferred by carbapenemases is considered to be more important than these mechanisms because carbapenemase-encoding genes are often located on mobile genetic elements and can be transferred to other bacteria (7).

Some previous studies have reported the occurrence of CPE in wastewater, including KPC-2-producing *Klebsiella* spp. and *Escherichia* spp. (8), KPC-2-producing *Klebsiella pneumoniae* (9), and NDM-1-positive *Escherichia coli* (10). However, compared with clinical isolates, data are limited for CPE in wastewater with respect to detailed genetic characteristics, such as fine-scale within-species phylogeny and virulence gene profiles. Detailed characterization of environmental CPE is important to better understand the molecular epidemiology and reservoirs of these clinically important microbes.

The present study was conducted to characterize CPE isolates in wastewater collected from municipal WWTPs and hospitals in Japan and Taiwan. Japan and Taiwan are not geographically distant, but the prevalences of carbapenemase-encoding genes in clinical *Enterobacteriaceae* isolates seem to be different in the two locales. Previous studies reported that Japanese CPE usually carry bla_{IMP} genes (11), whereas Taiwanese CPE carry a variety of carbapenemase-encoding genes, including bla_{KPC}, bla_{NDM}, bla_{IMP}, and bla_{VIM} (12). However, the prevalences of carbapenemase-encoding genes in CPE beyond clinical settings are not well understood in the two regions. In the present study, we performed whole-genome sequencing and analysis on CPE in wastewater to provide genetic information about resistance determinants, genetic contexts of carbapenemase-encoding genes, fine-scale phylogenies, and virulence gene profiles. Such information will help clarify the relatedness of environmental and clinical isolates and the mechanisms of spread of carbapenemase-encoding genes.

RESULTS AND DISCUSSION

Detection of CPE in wastewater. In total, 40 water samples were collected during the study period. CPE isolates were obtained from samples taken from a municipal WWTP in Taiwan, hospital wastewater in Taiwan, and a municipal WWTP in Japan. No CPE isolates were obtained from hospital wastewater samples in Japan. This may be because samples from the Japanese hospital consisted of untreated wastewater taken from sewer pipes and contained fecal material from fewer persons than the samples from the other locations, which consisted of treated wastewater (a mixture of fecal material from the population). We noted that some colonies showing *Enterobacteriaceae* profiles on chromID CARBA (bioMérieux, Marcy-l'Étoile, France) plates were oxidase positive, and we also detected such oxidase-positive *Enterobacteriaceae*-like colonies in samples from which we could not isolate CPE strains, including hospital wastewater samples in Japan. A previous study using chromID CARBA SMART for analyzing environmental samples also reported a high frequency of isolation of oxidase-positive microorganisms, such as *Pseudomonas* spp. (13). We could not determine the number of CPE in each sample because of the presence of these oxidase-positive *Enterobacteriaceae*-like colonies.

A total of 46 isolates were obtained. As described above, these isolates were obtained from samples taken from a municipal WWTP and hospital wastewater in Taiwan and a municipal WWTP in Japan, all consisting of biologically treated wastewater. One isolate was identified as redundant (i.e., isolated from the same sample, belonging to the same species and the same sequence type [ST], and carrying the same antimicrobial resistance genes). We excluded this redundant isolate, leaving 3 isolates from municipal WWTP samples from Taiwan, 32 isolates from hospital wastewater samples from Taiwan, and 10 isolates from municipal WWTP samples from Japan for further analysis. The species composition of these 45 isolates was as follows: *E. coli*,

TABLE 1 Species composition and carbapenemase-encoding genes of *Enterobacteriaceae* isolates

Carbapenemase-encoding gene (*n*)	Sample type[a] (*n*)				
	E. coli (*n* = 10)	*Klebsiella* spp. (*n* = 22)	*E. cloacae* complex (*n* = 10)	*Citrobacter* spp. (*n* = 2)	*R. ornithinolytica* (*n* = 1)
bla_{GES-5} (20)	TH (6)	JW (1), TW (1), TH (10)	TH (1)		JW (1)
bla_{GES-6} (7)	TH (1)	TW (1), TH (4)		TH (1)	
bla_{GES-24} (1)		JW (1)			
bla_{NDM-5} (3)	TH (3)				
bla_{IMP-8} (2)			TH (2)		
bla_{IMP-19} (1)		JW (1)			
bla_{KPC-2} (2)		TH (1)	TH (1)		
bla_{VIM-1} (1)		TH (1)			
bla_{GES-5} + bla_{IMP-8} (1)				TH (1)	
ND[b] (7)		TW (1)	JW (6)		

[a]JW, Japanese municipal WWTP; TW, Taiwanese municipal WWTP; TH, Taiwanese hospital wastewater.
[b]ND, no carbapenemase-encoding genes were detected.

n = 10; *Klebsiella* spp., *n* = 22; *Enterobacter cloacae* complex, *n* = 10; *Citrobacter* spp., *n* = 2; and *Raoultella ornithinolytica*, *n* = 1 (Table 1). Species identified by the MALDI (matrix-assisted laser desorption ionization) Biotyper Compass 4.1 (Bruker Daltonics GmbH, Bremen, Germany) and by genome sequence-based methods (average nucleotide identity [ANI] and digital DNA-DNA hybridization [DDH] calculation) were congruent in most cases, but the genome sequence-based methods were more accurate in determining the phylogenetic groups of *Klebsiella* spp. (KpI, KpII-A, KpII-B, and KpIII) and the *E. cloacae* complex (phylogenetic groups A to R). Data Set S1 in the supplemental material summarizes the genetic characteristics (sequence type, resistance genes, genetic context of carbapenemase-encoding genes, and plasmid replicons associated with carbapenemase-encoding genes), assembly statistics, and antibiotic susceptibility of each isolate. It should be noted that we did not collect isolates randomly from each plate but selected colonies based on their morphologies to represent the genetic diversity of bacterial strains in a sample. Moreover, only a limited number of colonies were picked from each plate. Therefore, the actual species composition and clonal composition of CPE isolates in the wastewater samples may differ from those that we observed in the present study, and the diversity of CPE is likely to be underestimated.

Phenotypic and genotypic resistance. The carbapenemase-encoding genes detected in the present study were as follows: bla_{GES-5} (*n* = 21), bla_{GES-6} (*n* = 7), bla_{IMP-8} (*n* = 3), bla_{NDM-5} (*n* = 3), bla_{KPC-2} (*n* = 2), bla_{GES-24} (*n* = 1), bla_{IMP-19} (*n* = 1), and bla_{VIM-1} (*n* = 1) (Table 1). One *Citrobacter freundii* isolate carried both bla_{GES-5} and bla_{IMP-8}. Seven isolates did not carry any carbapenemase-encoding genes, but they carried class C β-lactamases. AmpC β-lactamase overproduction and decreased outer membrane protein expression combined with an active efflux pump can contribute to carbapenem resistance in *Enterobacteriaceae* (7, 14), which may explain the carbapenem resistance in these seven isolates. All but one (bla_{IMP-19}-carrying JSWP033) of the CPE isolates from municipal WWTP samples carried bla_{GES} genes. The apparent high prevalence of bla_{GES} genes among our municipal WWTP isolates indicates that bla_{GES}-harboring microbes may be prevalent in the intestinal tracts of the sampled local populations in Japan and Taiwan, because bacterial isolates in wastewater may serve as representatives of the strains present within the human population in a given locale (15, 16). bla_{GES-5} was the most prevalent carbapenemase-encoding gene among the Taiwanese hospital wastewater isolates, despite the apparent absence of bla_{GES} among patients in Taiwan (12). A previous study similarly reported that bla_{GES-5} was prevalent among CPE isolated from hospital wastewater despite the absence of the gene among screening and clinical isolates within the hospital (17). The authors concluded that this may have been because unidentified carriers within the hospital were a reservoir for GES-5 or GES-5-positive *Enterobacteriaceae* were adapted to the environment and consistently present within the wastewater pipework. These possibilities may also apply to our study. In addition, there is potential for underestimation of the occurrence of bla_{GES} among

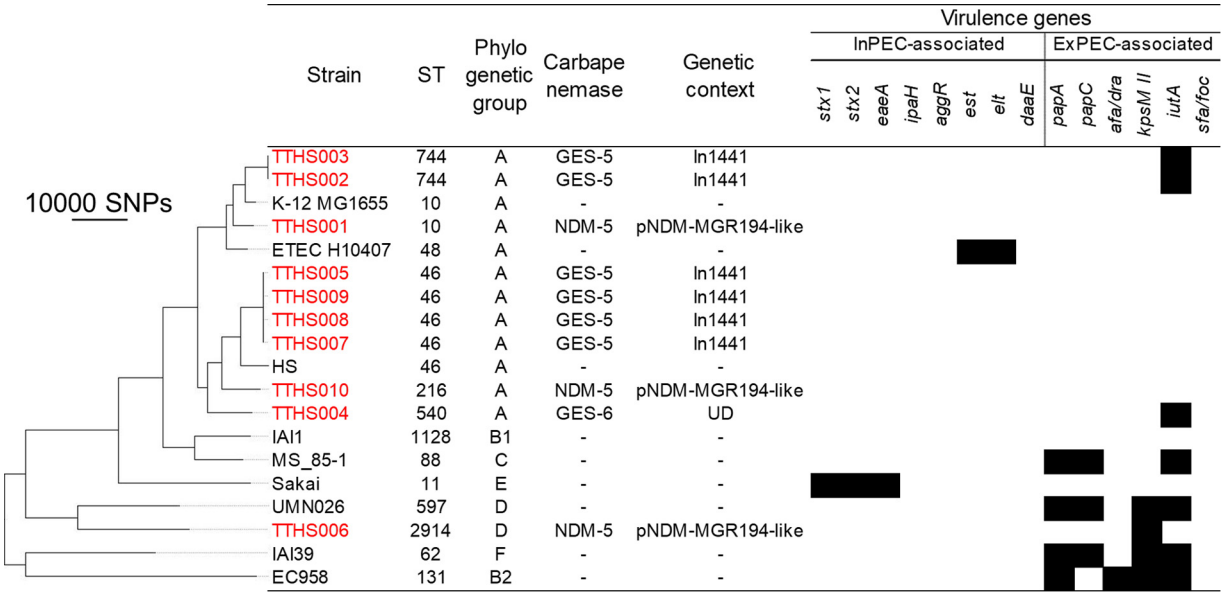


FIG 1 Phylogenetic tree of *E. coli* isolates. The tree was visualized using FigTree (http://tree.bio.ed.ac.uk/software/figtree/). In total, 181,286 SNP positions were identified, 107,866 of which occurred in at least 80% of the genomes and were used for the tree construction. UD indicates the genetic context of a carbapenemase-encoding gene was not determined for the isolate. In1441 contained a bla_{GES-5}-bla_{OXA-17} cassette array. The dashes in the carbapenemase and genetic context columns indicate the absence of carbapenemase-encoding genes. The black blocks represent the presence of a virulence gene. No chromosomal *ampC* promoter/attenuator mutations that can result in *ampC* overexpression were found among these *E. coli* isolates. Strains from Taiwanese hospital wastewater begin with TTHS and are colored red. The other strains are reference strains.

clinical isolates because MICs of carbapenems tended to be lower for the isolates harboring bla_{GES} and bla_{GES}-harboring isolates can be falsely negative in the Carba NP test, as discussed below. There were also CPE isolates from Taiwanese hospital wastewater carrying bla_{VIM-1}, bla_{NDM-5}, bla_{IMP-8}, and bla_{KPC-2}, findings consistent with a variety of carbapenemases recovered from patients in Taiwan (12).

Carbapenemase activity was confirmed in all isolates harboring bla_{VIM-1}, bla_{NDM-5}, bla_{IMP-8}, bla_{IMP-19}, or bla_{KPC-2} by the Carba NP test, whereas isolates harboring bla_{GES} genes and isolates without carbapenemase-encoding genes were negative (or invalid for one bla_{GES-5}-harboring isolate) by the Carba NP test (see Data Set S1 in the supplemental material). A previous study reported that *Enterobacteriaceae* harboring GES-5 can be falsely negative in the Carba NP test (18). MICs of imipenem and meropenem tended to be higher for isolates harboring metallo-beta-lactamase-encoding genes (bla_{VIM-1}, bla_{NDM-5}, bla_{IMP-8}, and bla_{IMP-19}) or bla_{KPC-2} than for those harboring bla_{GES} genes (see Fig. S1 in the supplemental material). In fact, all but one isolate (bla_{IMP-8}-harboring TTHS028) with these metallo-beta-lactamase-encoding genes or bla_{KPC-2} were nonsusceptible to imipenem or meropenem, while more than half of the bla_{GES}-harboring isolates were susceptible to both agents. These results may reflect the relatively weak carbapenemase activity of GES enzymes (19). Importantly, all isolates carried resistance genes other than carbapenemase-encoding genes (see Data Set S1 in the supplemental material). Although some genes seem to be chromosomally encoded and core to the species/genus (e.g., *fosA*, *oqxAB*, and the β-lactamase genes bla_{SHV}, bla_{OKP}, and bla_{LEN} in KpI, KpII, and KpIII [20]), the observed high prevalence of genes conferring resistance to different classes of antibiotics among CPE is of great concern.

Genetic contexts of carbapenemase-encoding genes. Among the 38 CPE isolates, we could determine the genetic contexts of carbapenemase-encoding genes in 32 isolates (Fig. 1 to 3; see also Data Set S1 in the supplemental material for the genetic context of the carbapenemase-encoding genes in each isolate). For the remaining six isolates, the genetic contexts could not be determined due to the limitations associated with short-read sequencing.

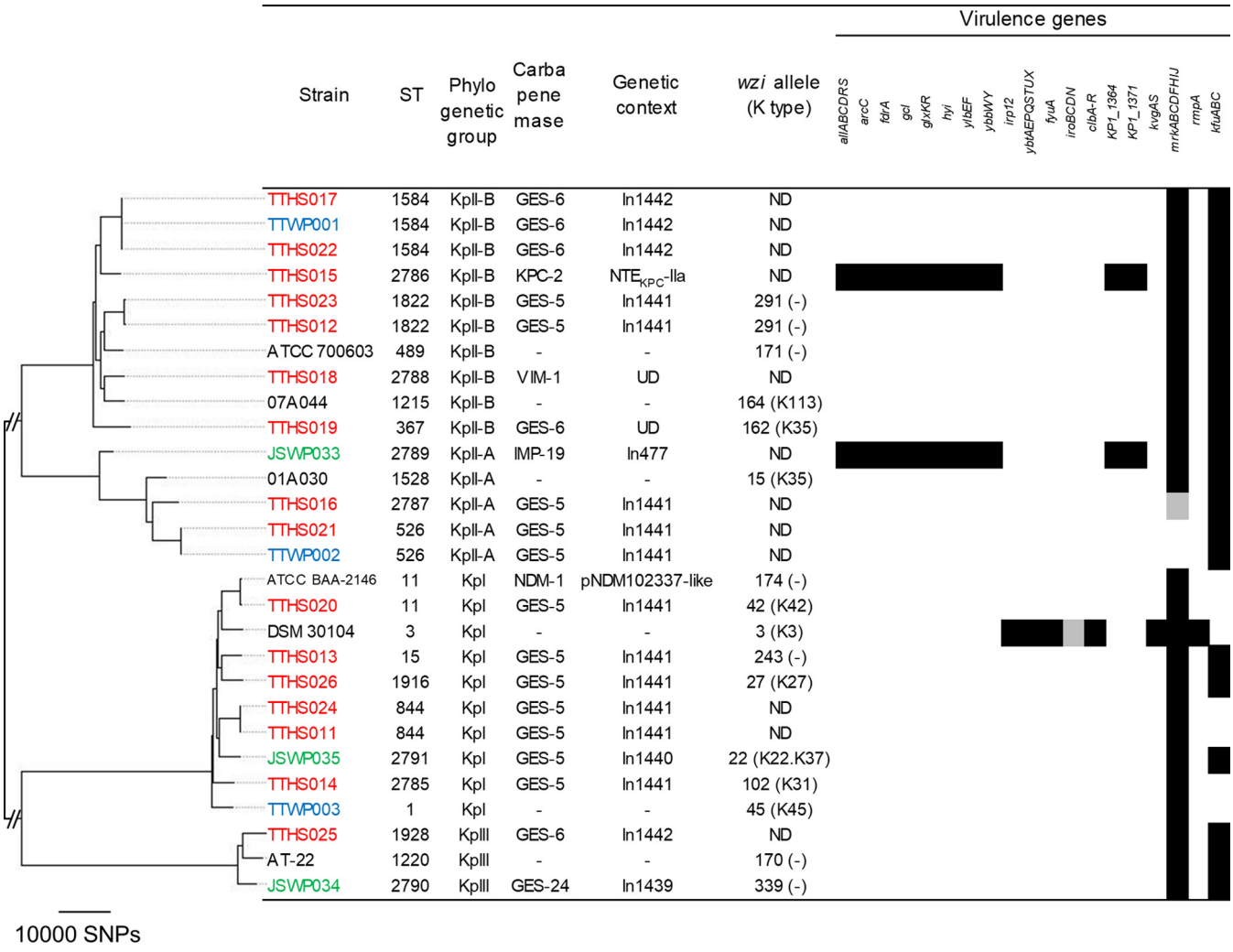
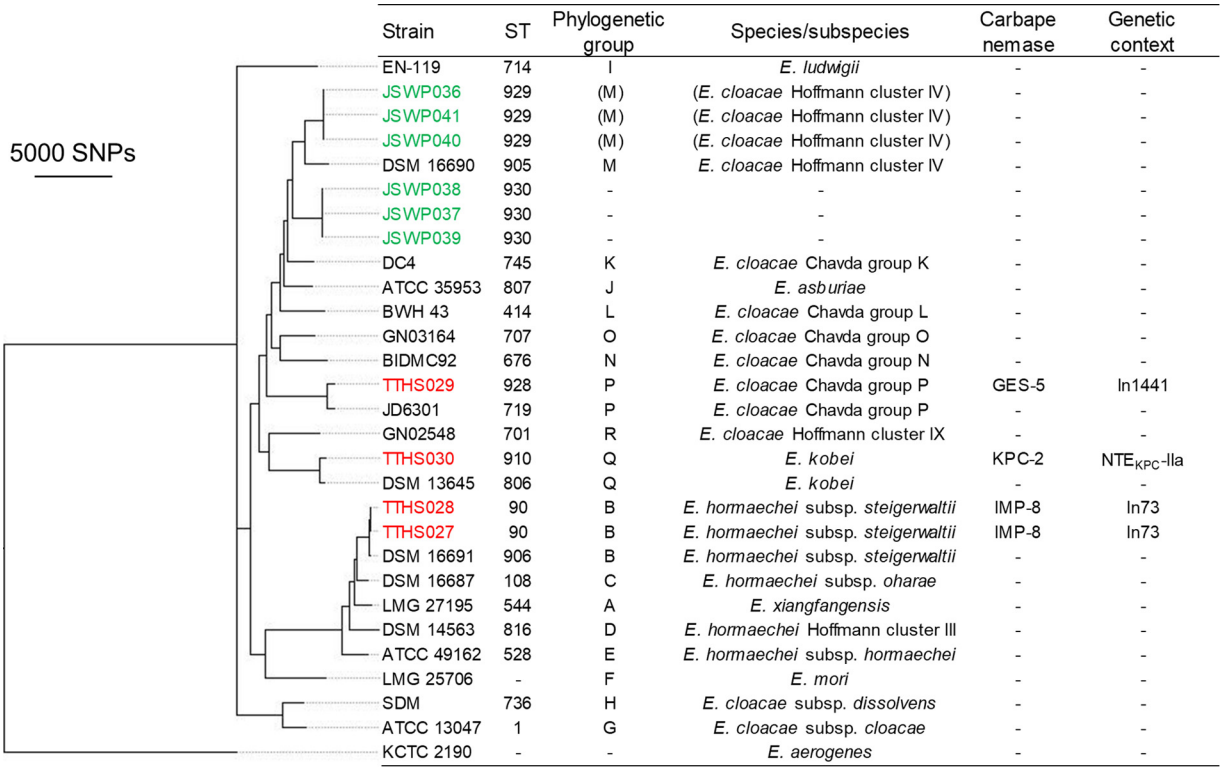


FIG 2 Phylogenetic tree of *Klebsiella* isolates. In total, 402,516 SNP positions were identified, 100,591 of which occurred in at least 80% of the genomes and were used for the tree construction. KpI corresponds to *K. pneumoniae*, KpII-A corresponds to *K. quasipneumoniae* subsp. *quasipneumoniae*, KpII-B corresponds to *K. quasipneumoniae* subsp. *similipneumoniae*, and KpIII corresponds to *K. variicola*. ST2785 to ST2791 are novel sequence types found in this study. UD indicates the genetic context of a carbapenemase-encoding gene was not determined for the isolate. In477 and In1439 to In1442 contained the following cassette arrays: bla_{IMP-19}-*aacA31*-bla_{OXA-21}-*aadA1* (In477), bla_{GES-24}-*aacA4* (In1439), bla_{GES-5}-*aacA31*-*catB8*-*aadA5* (In1440), bla_{GES-5}-bla_{OXA-17} (In1441), and bla_{GES-6}-*aacA4*-bla_{OXA-17} (In1442). NTE_{KPC} indicates a bla_{KPC}-bearing non-Tn*4401* element. In ATCC BAA-2146, Tn*125* was truncated at IS*Aba125* upstream of bla_{NDM-1} and at IS*CR27*. The dashes in the carbapenemase and genetic context columns indicate the absence of carbapenemase-encoding genes. ND indicates the *wzi* allele was not determined for the isolate. K types were not assigned for some *wzi* alleles (indicated by the dashes in parentheses), and only the *wzi* alleles are shown for these isolates. The black blocks represent the presence of a virulence gene/gene cluster. The gray blocks represent the presence of some genes in a gene cluster. Virulence genes of *iucABCD*, *iutA*, *mceABCDEGHIJ*, and *rmpA2* were sought but not found. Strains from Taiwanese hospital wastewater begin with TTHS and are colored red. Strains from the Taiwanese municipal WWTP begin with TTWP and are colored blue. Strains from the Japanese municipal WWTP begin with JSWP and are colored green. The other strains are reference strains.

bla_{GES} genes were situated within novel class 1 integrons. In1441, with a gene cassette array of bla_{GES-5}-bla_{OXA-17}, was prevalent among *E. coli* ($n = 6$), *Klebsiella* spp. ($n = 11$), and *E. cloacae* complex ($n = 1$) isolates from the Taiwanese municipal WWTP and hospital wastewater. In1442, with a gene cassette array of bla_{GES-6}-*aacA4*-bla_{OXA-17}, was found in *Klebsiella* isolates ($n = 4$) from the Taiwanese municipal WWTP and hospital wastewater. These findings suggest the circulation of the same integron among different species and in both community and clinical settings in Taiwan. In all of the gene cassette arrays mentioned above, the *attC* site of the bla_{OXA-17} gene cassette was interrupted by IS*Pa25*, which contains a putative transposase gene (*orf4*). Interestingly, the nucleotide sequence of this fused gene cassette, containing bla_{OXA-17} and *orf4*, shared 100% nucleotide identity with those in previously characterized class 1 integrons of *Pseudomonas aeruginosa* from Taiwan (21) and *K. pneumoniae* from



Strain	ST	Phylogenetic group	Species/subspecies	Carbapenemase	Genetic context
EN-119	714	I	*E. ludwigii*	-	-
JSWP036	929	(M)	(*E. cloacae* Hoffmann cluster IV)	-	-
JSWP041	929	(M)	(*E. cloacae* Hoffmann cluster IV)	-	-
JSWP040	929	(M)	(*E. cloacae* Hoffmann cluster IV)	-	-
DSM 16690	905	M	*E. cloacae* Hoffmann cluster IV	-	-
JSWP038	930	-	-	-	-
JSWP037	930	-	-	-	-
JSWP039	930	-	-	-	-
DC4	745	K	*E. cloacae* Chavda group K	-	-
ATCC 35953	807	J	*E. asburiae*	-	-
BWH 43	414	L	*E. cloacae* Chavda group L	-	-
GN03164	707	O	*E. cloacae* Chavda group O	-	-
BIDMC92	676	N	*E. cloacae* Chavda group N	-	-
TTHS029	928	P	*E. cloacae* Chavda group P	GES-5	In1441
JD6301	719	P	*E. cloacae* Chavda group P	-	-
GN02548	701	R	*E. cloacae* Hoffmann cluster IX	-	-
TTHS030	910	Q	*E. kobei*	KPC-2	NTE_{KPC}-IIa
DSM 13645	806	Q	*E. kobei*	-	-
TTHS028	90	B	*E. hormaechei* subsp. *steigerwaltii*	IMP-8	In73
TTHS027	90	B	*E. hormaechei* subsp. *steigerwaltii*	IMP-8	In73
DSM 16691	906	B	*E. hormaechei* subsp. *steigerwaltii*	-	-
DSM 16687	108	C	*E. hormaechei* subsp. *oharae*	-	-
LMG 27195	544	A	*E. xiangfangensis*	-	-
DSM 14563	816	D	*E. hormaechei* Hoffmann cluster III	-	-
ATCC 49162	528	E	*E. hormaechei* subsp. *hormaechei*	-	-
LMG 25706	-	F	*E. mori*	-	-
SDM	736	H	*E. cloacae* subsp. *dissolvens*	-	-
ATCC 13047	1	G	*E. cloacae* subsp. *cloacae*	-	-
KCTC 2190	-	-	*E. aerogenes*	-	-

FIG 3 Phylogenetic tree of *E. cloacae* complex isolates. In total, 1,321,589 SNP positions were identified, 24,510 of which occurred in at least 80% of the genomes and were used for the tree construction. *Enterobacter aerogenes* KCTC 2190 was used as an outgroup. Hoffmann clusters are genetic clusters identified by Hoffmann and Roggenkamp based most exhaustively on *hsp60* sequences (64). ST928, ST929, and ST930 are novel sequence types identified in the present study. The dashes in the ST, phylogenetic group, and species/subspecies columns indicate that these properties could not be assigned to the corresponding isolates. In73 contained a bla_{IMP-8}-*aacA4-catB3* cassette array, and In1441 contained a bla_{GES-5}-bla_{OXA-17} cassette array. NTE_{KPC} is a bla_{KPC}-bearing non-Tn*4401* element. The dashes in the carbapenemase and genetic context columns indicate the absence of carbapenemase-encoding genes. Strains from the Taiwanese hospital wastewater begin with TTHS and are colored red. Strains from the Japanese municipal WWTP begin with JSWP and are colored green. The other strains are reference strains.

Korea (22). On the other hand, different novel integrons without this fused gene cassette were found among bla_{GES}-harboring Japanese isolates, namely, In1439 (cassette array, bla_{GES-24}-*aacA4*) in one KpIII isolate and In1440 (cassette array, bla_{GES-5}-*aacA31-catB8-aadA5*) in one KpI isolate. We were able to determine the promoter types and downstream structures in 8 and 12 bla_{GES}-containing integrons, respectively (see also Data Set S1 in the supplemental material). Only strong promoters, namely, PcW_{TGN-10} ($n = 5$), $PcH2_{TGN-10}$ ($n = 2$), and PcS ($n = 1$), were detected. Of the 12 integrons for which we could determine downstream structures, all carried 3′-conserved segment (CS) structures immediately downstream of the gene cassettes. Of these, 3′-CS-IS*6100* (with or without a 1-bp deletion in IS*6100*) was the most common ($n = 9$).

bla_{IMP} genes were situated in previously reported class 1 integrons. Two *E. cloacae* complex isolates from Taiwanese hospital wastewater carried In73 with a gene cassette array of bla_{IMP-8}-*aacA4-catB3*. This integron was previously found in a clinical isolate of *K. pneumoniae* (23) and clinical isolates of the *E. cloacae* complex (24) obtained in Tainan City, Taiwan. One KpII-A isolate from the Japanese municipal WWTP carried In477 with a gene cassette array of bla_{IMP-19}-*aacA31*-bla_{OXA-21}-*aadA1*. This integron was previously found in clinical isolates of *Acinetobacter* spp. in the Kansai region of Japan (25). These results are interesting because the same integrons were found among clinical isolates obtained in the same regions as the present study. We were able to determine the promoter type and the downstream structure of the bla_{IMP-19}-containing integron of the Japanese isolate (see Data Set S1 in the supplemental material). This integron contained a PcS strong promoter and carried 3′-CS-IS*6100* immediately downstream of the gene cassettes.

bla$_{NDM-5}$ was detected in three *E. coli* isolates from Taiwanese hospital wastewater. *bla*$_{NDM-5}$ was carried by an IS*26*-like insertion sequence (contig break in the transposase gene)-*dsbC-trpF-ble*$_{MBL}$-*bla*$_{NDM-5}$-IS*Aba125* (interrupted by IS*5*) genetic element. For all three isolates, PlasmidFinder detected an IncX3 replicon in *bla*$_{NDM-5}$-containing contigs. Further analysis revealed that the *bla*$_{NDM-5}$-containing contigs were highly similar (≥99% sequence identity over ≥90% of the length) to a previously described IncX3 plasmid, pNDM-MGR194 of *K. pneumoniae* MGR-K194 in India (26). Plasmids that are identical or nearly identical to plasmid pNDM-MGR194 have been reported among *E. coli* isolates from China, Australia, and Denmark (27).

bla$_{KPC-2}$ was found in one KpII-B isolate and one *E. cloacae* complex isolate from Taiwanese hospital wastewater. In both cases, the gene occurred in the context of the *bla*$_{KPC}$-bearing non-Tn*4401* element IIa (28). An IncP6 replicon was detected in the *bla*$_{KPC-2}$-carrying contig of the KpII-B isolate, whereas no plasmid replicons were detected in that of the *E. cloacae* complex isolate.

Phylogenetic characteristics and virulence potential. The *E. coli* isolates were detected only in Taiwanese hospital wastewater and belonged to six different STs (Fig. 1). ST46 ($n = 4$) was the most prevalent, followed by ST744 ($n = 2$) (note that these isolates belonging to the same ST were obtained from different samples and not redundant, as described above). Nine isolates belonged to phylogenetic group A, which is usually associated with intestinal pathogenic *E. coli* (InPEC) or commensals (29). *E. coli* pathotyping based on the presence of virulence genes indicated that none of our CPE isolates were pathogenic, although three strains carried the extraintestinal pathogenic *E. coli* (ExPEC)-associated gene *iutA* and one strain carried another ExPEC-associated gene, *kpsM* II (carriage of at least two ExPEC-associated genetic markers is needed to assign an isolate to ExPEC).

Isolates classically identified as *K. pneumoniae* can be divided into KpI (*K. pneumoniae*), KpII-A (*Klebsiella quasipneumoniae* subsp. *quasipneumoniae*), KpII-B (*Klebsiella quasipneumoniae* subsp. *similipneumoniae*), and KpIII (*Klebsiella variicola*) (20, 30). The prevalence of these phylogenetic groups among the *Klebsiella* isolates in the present study was as follows: KpI, $n = 8$; KpII-A, $n = 4$; KpII-B, $n = 8$; and KpIII, $n = 2$ (Fig. 2). This prevalence is different from those observed in previous studies analyzing clinical isolates (31, 32), which reported a higher prevalence of KpI than of KpII and KpIII. This may primarily be because KpII and KpIII are associated more frequently with carriage (not being the cause of an infection), whereas KpI is associated with human infection (20, 33). The most prevalent ST was ST1584 ($n = 3$), followed by ST526 ($n = 2$), ST844 ($n = 2$), and ST1822 ($n = 2$). Clonal overlaps for ST1584 and ST526 were observed between the municipal WWTP and hospital wastewater isolates from Taiwan, suggesting that carbapenemase-producing KpII isolates belonging to these STs may be prevalent in both community and clinical settings in Taiwan. It is worrisome that successful multidrug-resistant clones, namely, ST11 and ST15 (31, 34), were found among CPE isolates in the present study. Among the virulence genes analyzed, the allantoinase gene cluster was found in two of the KpII isolates, *kfuABC* was found in all of the KpII and KpIII isolates and 38% of the KpI isolates, and *mrkABCDFHIJ* was found in all the isolates except some of the KpII-A isolates. These results are in agreement with a previous study (20). However, the genes *rmpA* and *rmpA2* and the siderophore clusters, which are significantly associated with invasive human infections among KpI isolates (20), were not detected among the *Klebsiella* isolates in the present study. The polysaccharide capsule is a key virulence determinant, and the K types K1, K2, K5, K20, K54, and K57 are known to be associated with liver abscesses and other community-acquired invasive infections (35). None of our isolates were identified as these K types by *wzi* typing.

By employing whole-genome analyses, Chavda et al. determined that *E. cloacae* complex isolates fall into 18 phylogenetic groups (A to R), each corresponding to a distinct species/subspecies (36). According to the phylogenetic tree in Fig. 3 and ANI and digital DDH analyses, four of our *E. cloacae* complex isolates could be assigned to

1 of these 18 phylogenetic groups, namely, group B ($n = 2$), group P ($n = 1$), or group Q ($n = 1$). Group B (*Enterobacter hormaechei* subsp. *steigerwaltii*) is one of the most prevalent phylogenetic groups among clinical *E. cloacae* complex isolates (37). Two bla_{IMP-8}-carrying strains (TTHS027 and TTHS028) belonging to this group were assigned to ST90, which is one of the STs found among clinical IMP-producing isolates (38). A fine-scale phylogenetic tree was constructed to gain insights into the phylogeny of the remaining six isolates (see Fig. S2 in the supplemental material). Three isolates (JSWP036, JSWP040, and JSWP041) seem to be closely related to group M (*E. cloacae* Hoffmann cluster IV), and the ANI values of these three strains and the type strain of group M were 95.8%, just slightly higher than 95%. However, the digital DDH estimate was 64.7%, indicating that the three strains may belong to different species than group M. The other three isolates (JSWP037, JSWP038, and JSWP039) showed ANI values of ≤95% among all of the type or reference strains of 18 phylogenetic groups, indicating that the three strains belong to another, unrecognized group. Further studies are needed to elucidate the phylogeny and determine the species of these six isolates.

Two *Citrobacter* isolates were obtained in the present study, one belonging to *C. freundii* and the other belonging to *Citrobacter amalonaticus*. The *C. freundii* isolate belonged to ST22. This ST was previously reported among carbapenemase-producing clinical *Citrobacter* isolates (39). We also detected one *R. ornithinolytica* isolate. Two recent studies reported carbapenemase-producing *Citrobacter* spp. and *R. ornithinolytica* isolates in wastewater and surface water, but the detected isolates carried different types of carbapenemase-encoding genes (bla_{OXA}, bla_{VIM}, and bla_{NDM}) than those in the present study (bla_{GES} and bla_{IMP}) (40, 41). However, it should be noted that these two studies detected carbapenemase-encoding genes by PCR, and bla_{GES} and bla_{IMP} were not investigated. This highlights the strength of the whole-genome-sequencing approach, which enables the detection of all antibiotic resistance genes annotated in the database.

This study has some limitations. The number of isolates characterized ($n = 45$) is not large, and most isolates ($n = 32$) were from Taiwanese hospital wastewater (note that *E. coli* isolates were obtained only from Taiwanese hospital wastewater). Our collection does not represent the prevalence of CPE in wastewater in Japan and Taiwan because our isolates were obtained from a limited number of samples collected over relatively short periods. Moreover, we were not able to determine the genetic surroundings of carbapenemase-encoding genes in some isolates and could not resolve the plasmid structures due to the limitations associated with short reads. Further studies are needed, including a larger number of global isolates obtained from different types of WWTPs and employing long-read sequencing.

In the present study, we characterized, by whole-genome analysis, CPE isolates in wastewater in terms of resistance determinants, genetic contexts of carbapenemase-encoding genes, phylogeny, and virulence potential. The results indicate that CPE isolates with various carbapenemase-encoding genes in different genetic contexts are present in biologically treated wastewater and may disseminate into the environment. This study highlights the need to monitor for antibiotic-resistant bacteria in the environment, not only in clinical settings.

MATERIALS AND METHODS

Sample collection and isolation of CPE. Ten wastewater samples per location were collected from a municipal WWTP and a hospital in the Kansai region of Japan in October 2015 (42). Samples from the WWTP were collected from effluent from the final settling tanks after biological (activated-sludge) treatment, and samples from the hospital were taken from a sewer system (sewer pipes) and consisted of untreated wastewater. We also collected 10 biologically treated wastewater samples (secondary sedimentation tank effluent) per location from a municipal WWTP and a hospital WWTP in Tainan City, Taiwan, between August and September 2015. All the samples were collected in sterile 50-ml centrifuge tubes or sampling bottles, transported to the laboratory, and processed as soon as possible. A total of 40 water samples were processed by using membrane filtration methods with chromID CARBA agar (bioMérieux, Marcy-l'Étoile, France) for enumeration and isolation of CPE. For each sample, up to four colonies showing *E. coli* profiles (pink to burgundy) or KESC (*Klebsiella-Enterobacter-Serratia-Citrobacter*) group profiles (bluish green to bluish gray) on the chromID CARBA plate were isolated, respectively (care was taken to select colonies showing different morphologies, if possible). The isolates were restreaked

on fresh chromID CARBA plates and incubated until pure colonies were obtained. Isolates collected in Taiwan were stored in Casitone medium (Eiken, Tokyo, Japan) and transported to the laboratory in Japan. The oxidase test was performed using a cytochrome oxidase test strip (Nissui, Tokyo, Japan) for each isolate, and oxidase-negative isolates were stored at −85°C in 35% glycerol.

Species identification and antibiotic susceptibility testing. Species identification was performed with a MALDI Biotyper Compass 4.1. Susceptibility to 23 antibiotics was evaluated by microdilution using the dry-plate Eiken assay (Eiken, Tokyo, Japan) according to CLSI guidelines (43). Strains were checked for carbapenemase activity by the Carba NP test (44).

Genome sequencing and assembly. DNA was extracted from each isolate using a DNeasy blood and tissue kit (Qiagen, Hilden, Germany). DNA libraries were prepared using a Nextera XT DNA sample preparation kit (Illumina, San Diego, CA) and subsequently sequenced using Illumina MiSeq 300-bp paired-end sequencing technologies to achieve an average depth of coverage of 75. Raw reads generated from each sample were trimmed using ERNE-Filter (45) and assembled using SPAdes v3.10.0 (46). Assemblies were improved using Pilon (47). The assembled contigs were subjected to the analyses described below.

Phylogenetic analysis. Species identification was also performed for each isolate based on the genomic data. The ANI analysis was performed using JSpeciesWS (48). If an isolate shared an ANI value of >95% with a type strain (or a reference strain if the genome of the type strain was not available) of a certain species belonging to the family *Enterobacteriaceae*, the isolate was identified as that species (49). Digital DDH values were also calculated using formula 2 on the GGDC website to confirm the results (50).

Isolates identified as *E. coli*, *K. pneumoniae* (KpI), *K. quasipneumoniae* subsp. *quasipneumoniae* (KpII-A), *K. quasipneumoniae* subsp. *similipneumoniae* (KpII-B), *K. variicola* (KpIII), and *E. cloacae* complex (phylogenetic groups A to R) were analyzed with kSNP to construct whole-genome single nucleotide polymorphism (SNP)-based within-species/genus phylogenetic trees (51, 52). Selected isolates analyzed in other studies, i.e., isolates analyzed by Kaas et al. and Forde et al. for *E. coli* (53, 54), isolates analyzed by Brisse et al. and Hudson et al. for *Klebsiella* spp. (30, 55), and isolates analyzed by Chavda et al. for the *E. cloacae* complex (36), were also included in the trees to place our isolates in broader phylogenetic contexts. All the trees were parsimony trees and were constructed based on SNP loci occurring in at least 80% of the strains. STs were assigned to isolates using multilocus sequence type (MLST) databases (http://bigsdb.pasteur.fr/klebsiella/; http://pubmlst.org/ecloacae/; http://pubmlst.org/cfreundii/; http://mlst.ucc.ie/mlst/dbs/Ecoli/). *E. coli* phylogenetic groups were determined as described previously (56).

Identification of antimicrobial resistance determinants and virulence genes. Antimicrobial resistance genes were detected using the ResFinder antimicrobial resistance gene database (57) (with a threshold of 90% identity and a minimum length of 60%) and NCBI Beta-Lactamase Data Resources. For the *E. coli* isolates, chromosomal *ampC* promoter/attenuator mutations were analyzed as described previously (58). Contigs with carbapenemase-encoding genes were manually annotated to determine the genetic contexts of the genes. Integrons were classified according to INTEGRALL (http://integrall.bio.ua.pt/) (59). Pc-P2 promoters in class 1 integrons were analyzed according to a previous study (60). Insertion sequences were identified by using the ISfinder database (61). Plasmid replicons were detected using PlasmidFinder (62) (with a threshold of 80% identity and a minimum length of 60%).

For *E. coli* isolates, virulence genes were detected as described previously (42), and pathotypes were defined based on the presence of specific virulence genes (63). For *Klebsiella* isolates, virulence genes were detected using the Institut Pasteur *Klebsiella* database (http://bigsdb.pasteur.fr/klebsiella), and the K capsular type was determined based on *wzi* alleles (35). Presence of a virulence gene was arbitrarily defined as >80% sequence identity over >80% of the length of the reference gene.

Accession number(s). The genome sequence data obtained in the present study have been deposited in the DDBJ Sequence Read Archive database (DDBJ accession number DRA006131). The sequences of novel integrons found in this study are available under the following accession numbers: In1439, LC318533; In1440, LC318534; In1441, LC318535 and LC318536; and In1442, LC318537.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.02501-17.

SUPPLEMENTAL FILE 1, PDF file, 0.5 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.1 MB.

ACKNOWLEDGMENTS

We thank the team of curators of the Institut Pasteur MLST and whole-genome MLST databases for curating the data and making them publicly available at http://bigsdb.pasteur.fr. We thank Tohru Miyoshi-Akiyama for curating the *Enterobacter cloacae* MLST databases. We thank Thomas Jové from INTEGRALL for curating the integrons.

This work was supported by the Kyoto University Foundation, the Kurita Water and Environment Foundation, and the River Fund of the River Foundation, Japan. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

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