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Occurrence of Clinically Important Lineages, Including the Sequence Type 131 C1-M27 Subclone, among Extended-Spectrum-β-Lactamase-Producing *Escherichia coli* in Wastewater

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ABSTRACT Contamination of environmental waters by extended-spectrum-β-lactamase (ESBL)-producing *Escherichia coli* (ESBLEC) is of great concern. Wastewater treatment plants (WWTPs) and hospitals release large amounts of ESBLEC into the environment. In the present study, we isolated ESBLEC strains from wastewater collected from a WWTP and a hospital in Japan and performed whole-genome sequencing to characterize these strains. Genomic analysis of 54 strains (32 from the WWTP and 22 from hospital wastewater) revealed the occurrence of clinically important clonal groups with extraintestinal pathogenic *E. coli* status in the WWTP and hospital wastewater. Fine-scale phylogenetic analysis was performed to further characterize 15 sequence type 131 (ST131) complex strains (11 from the WWTP and 4 from hospital wastewater). These ST131 complex strains were comprised of the following different subgroups: clade A ($n = 2$), C1-M27 ($n = 8$), and C1 (non-C1-M27) ($n = 1$) for strains from the WWTP and clade A ($n = 2$), C1-M27 ($n = 1$), and C1 (non-C1-M27) ($n = 1$) for strains from hospital wastewater. The results indicate that ESBLEC strains belonging to clinically important lineages, including the C1-M27 clade, may disseminate into the environment through wastewater, highlighting the need to monitor for antibiotic resistance in wastewater.

KEYWORDS ESBL, *Escherichia coli*, wastewater, whole-genome sequencing

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The occurrence of antibiotic-resistant bacteria in the environment increases global health risks. Of great concern is extended-spectrum-β-lactamase (ESBL)-producing *Escherichia coli* (ESBLEC) because some *E. coli* strains are pathogenic (1) and treatment options are limited for ESBLEC infections (2). Human intestinal carriage of ESBLEC is well documented in community and clinical settings (3, 4). Therefore, wastewater treatment plants (WWTPs) and hospitals are considered to be major sources of ESBLEC released into the environment (5). In fact, the presence of ESBLEC in hospital wastewater and the inflow and outflow of WWTPs has been well documented in previous studies (6–8). However, data are limited with respect to detailed genetic characteristics of ESBLEC in wastewater.

Clinical ESBLEC isolates have often been characterized at the sequence type (ST) level by using multilocus sequence typing (MLST), and some pandemic clonal lineages of ESBLEC have been identified in this way. Among them, a clonal lineage of ST131 is of particular concern because this clone is the predominant lineage among drug-resistant extraintestinal pathogenic *E. coli* (ExPEC) strains worldwide (9, 10). Recent studies based on whole-genome sequencing showed that ST131 can be divided into

three clades, namely, A/*H*41, B/*H*22, and C/*H*30 (11, 12). *H*41, *H*22, and *H*30 indicate the *fimH* (type 1 fimbrial adhesin gene) allele type, and most strains belonging to each clade are known to carry the corresponding *fimH* allele. Clade C contains C0, C1, and C2, which can be defined based on the positions in the whole-genome phylogeny (13). Previous studies reported that the $bla_{CTX\text{-}M\text{-}15}$-harboring C2/*H*30Rx is highly responsible for the pandemic of ExPEC strains that carry ESBLs (9, 11, 12). Recently, we described a novel ST131 C1 subclade with $bla_{CTX\text{-}M\text{-}27}$, named C1-M27, by analysis of clinical ST131 strains, and we determined that this subclade is prevalent among Japanese ESBL-producing ST131 isolates and is also contributing to the global spread of ST131 (14). Importantly, some previous studies have detected ESBLEC belonging to ST131 in wastewater (15, 16). Dolejska et al. (15) detected CTX-M-15-producing *E. coli* strains belonging to B2-O25b-ST131 in treated wastewater, and Colomer-Lluch et al. (16) reported the presence of CTX-M-15-producing O25b:H4-B2-ST131 strains in raw urban sewage. However, those studies did not perform a whole-genome single nucleotide polymorphism (SNP)-based phylogenetic analysis. In fact, limited information exists regarding the fine-scale phylogeny of environmental ST131 strains. In particular, the reports describing *E. coli* belonging to C1-M27 are almost entirely restricted to clinical isolates (14). Detection and characterization of environmental *E. coli* strains belonging to this clade are needed to better understand the molecular epidemiology and reservoirs of the C1-M27 clade.

In the present study, we performed whole-genome sequencing and analysis of ESBLEC isolated from wastewater collected in Japan to examine the genetic characteristics of the detected strains and determine the presence of clinically important ESBLEC lineages, including the C1-M27 clade, in wastewater. A whole-genome approach was adopted because it enables us to obtain comprehensive information on genetic characteristics, such as virulence gene profiles, antibiotic resistance determinants, and fine-scale phylogeny.

RESULTS AND DISCUSSION

Detection and isolation of ESBLEC. During the study period, we collected 10 samples from the WWTP and 10 samples from hospital wastewater. All samples tested positive for *E. coli* and ESBLEC (see Table S1 in the supplemental material for concentrations of total *E. coli* and ESBLEC CFU in each sample). The average proportion of ESBLEC CFU among total *E. coli* CFU was 4.2% (minimum, 2.3%; maximum, 9.3%) for the WWTP samples, which is relatively high compared to previous studies (0.4% to 2.3%) (7). Conversely, the average proportion was 3.5% (minimum, 0.2%; maximum, 11.3%) for the hospital wastewater, and this value is relatively low compared to previous studies (3.8% to 13.6%) (7). Wastewater treatment processes can increase the proportion of resistant bacteria because the presence of antibiotics used in human medicine in wastewater poses selective pressures and the high cell density sustained by a nutrient-rich environment can promote the transfer of antibiotic resistance genes (5, 17). These may be reasons for the relatively high proportion of ESBLEC CFU in the WWTP samples.

In total, 32 strains from the WWTP and 29 strains from the hospital wastewater were isolated. Four strains were identified as *Citrobacter freundii* (genomic analysis also confirmed this), and three strains were identified as redundant strains (i.e., strains isolated from the same sample, belonging to the same ST, and carrying the same antimicrobial resistance genes). Therefore, we removed these seven strains, leaving 32 strains from the WWTP and 22 strains from the hospital wastewater for further analysis (see Data Set S1 in the supplemental material for information on these 54 strains).

Phylogenetic analysis. A whole-genome SNP-based tree was constructed using kSNP3 (Fig. 1). ESBLEC strains are scattered throughout the phylogenetic tree, representing seven phylogenetic groups (A, B1, B2, C, D, E, and F). MLST identified 28 STs, including two novel STs (ST7213 and ST7214). ST131 ($n = 10$) was the most prevalent ST among the WWTP isolates. One WWTP isolate belonged to ST7214, which is a

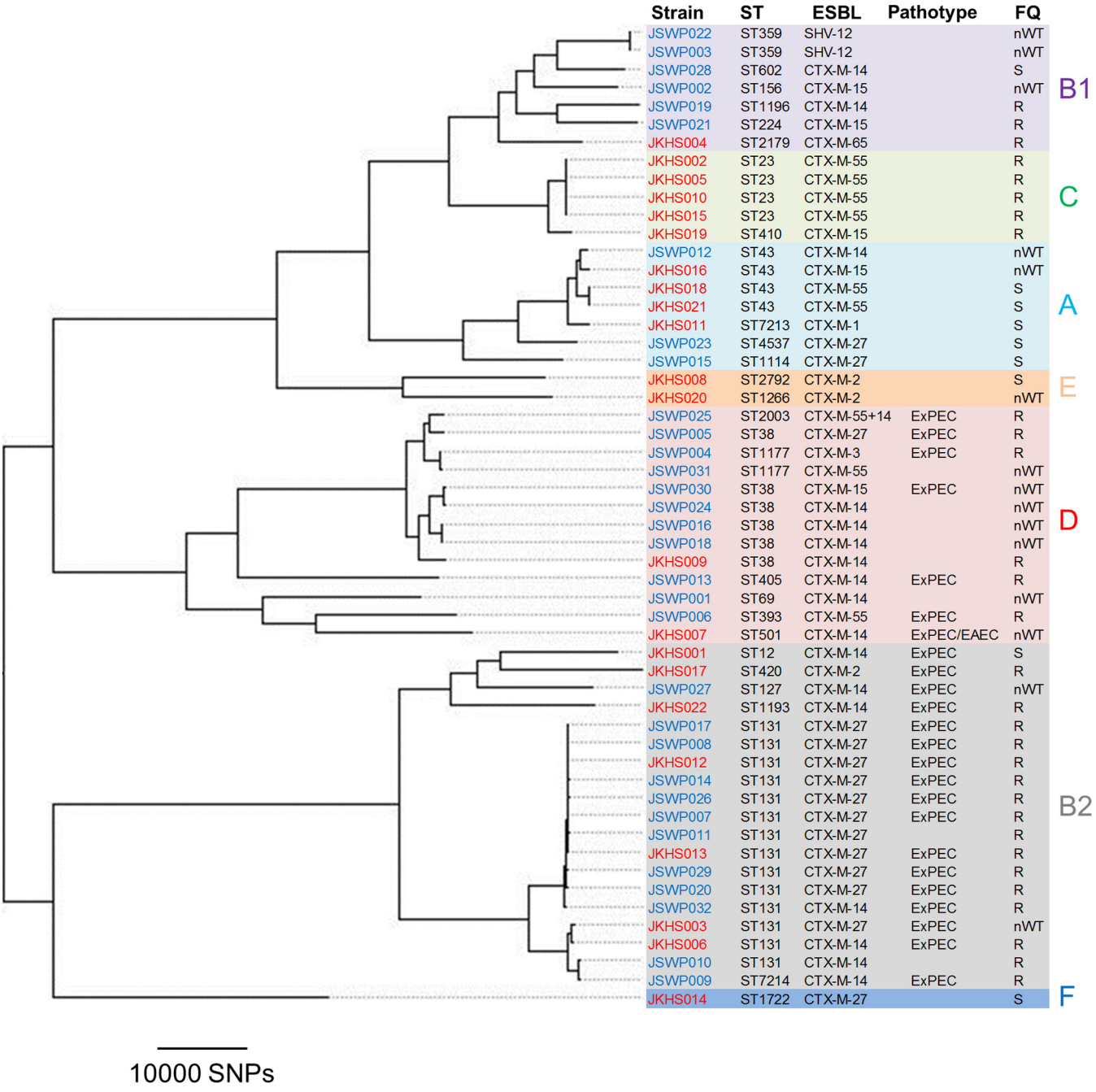


FIG 1 Phylogeny of 54 ESBLEC strains. A parsimony tree was constructed based on SNP loci occurring in at least 50% of the strains. The tree was visualized using FigTree (http://tree.bio.ed.ac.uk/software/figtree/). Colors of the strain names reflect the isolation sources, i.e., blue for the WWTP and red for hospital wastewater. Phylogenetic groups (A, B1, B2, C, D, E, and F) are indicated with different colors. The FQ column indicates ciprofloxacin susceptibilities for each isolate (S, susceptible; nWT, non-wild type according to the ECOFF criteria; R, resistant according to the clinical breakpoint).

single-locus variant (SLV) of ST131. ST131 ($n = 4$) and ST23 ($n = 4$) were the most prevalent STs among the strains obtained from the hospital wastewater. Clonal overlaps for ST38, ST43, and ST131 were observed between the WWTP and hospital wastewater isolates, suggesting that ESBLEC belonging to these STs may be prevalent in both community and clinical settings. It should be noted that we did not collect ESBLEC randomly from each plate but selected colonies based on their morphologies to represent the genetic diversity of ESBLEC strains in a sample. Therefore, the actual clonal compositions of ESBLEC isolates in the wastewater samples may differ from those that we observed in the present study.

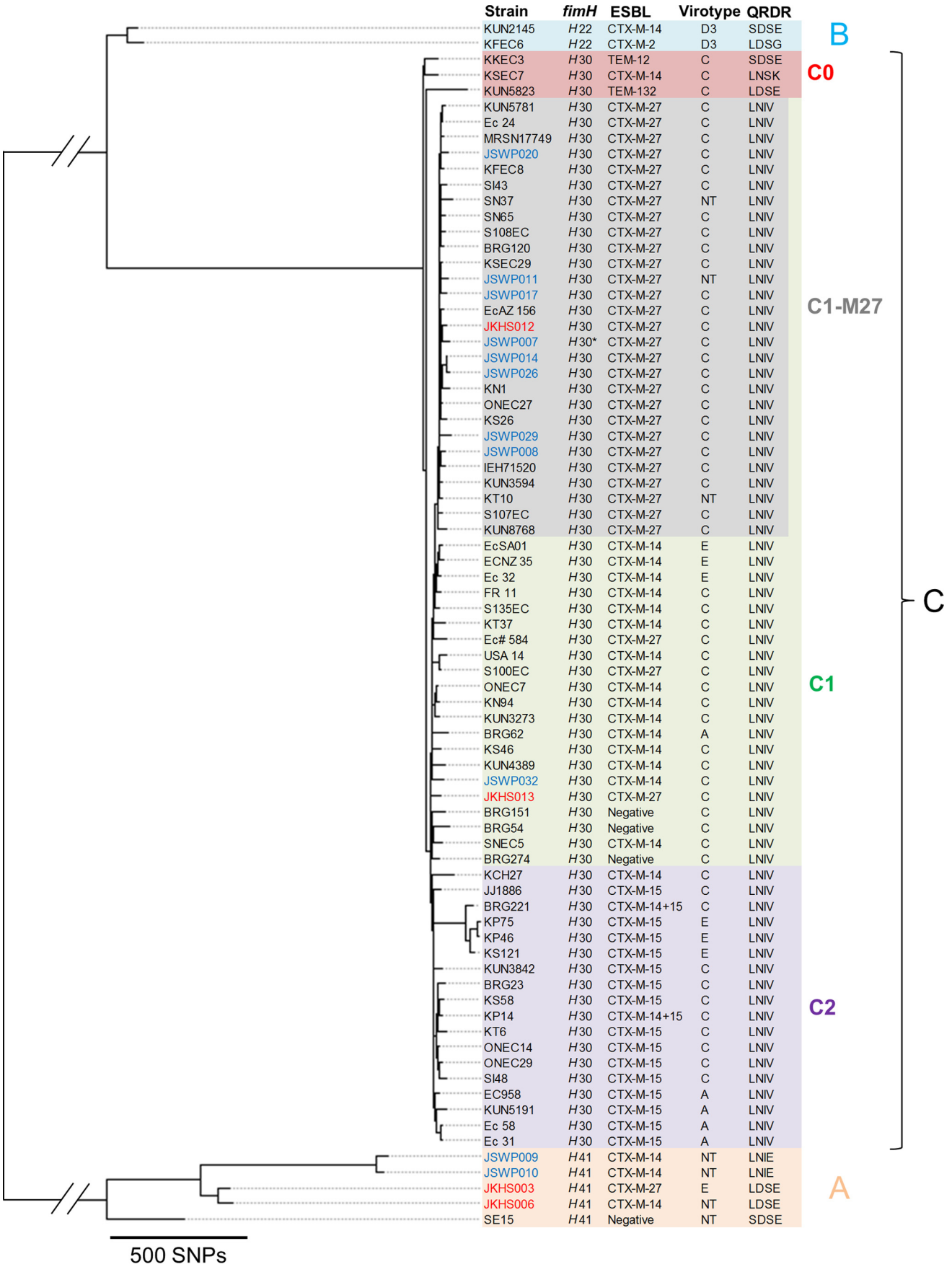
Pathotyping of the 54 strains revealed that 24 (44.4%) strains had ExPEC status. These ExPEC strains belonged to phylogenetic groups B2 and D, which is congruent with observations that these phylogenetic groups are associated with human extraintestinal infections (18). Furthermore, these ExPEC strains included those belonging to clinically important clonal groups, such as ST12, ST38, ST127, ST131, ST393, and ST405 (10), posing a public health concern. One of the 24 ExPEC strains, JKHS007, also had enteroaggregative *E. coli* (EAEC) status due to possession of *aggR*. A previous study reported human-derived *E. coli* belonging to ST38 with characteristics of both uropathogenic *E. coli* and EAEC (19). JKHS007 belonged to ST501, which is associated with EAEC from human sources, according to EnteroBase. Further analysis of sequence data revealed that JKHS007 carried ExPEC-associated genes, such as *sitABCD* (iron/manganese transport) as well as *iutA* and *kpsM II* (two of the five key markers defining ExPEC). The cooccurrence of ExPEC and EAEC-associated genes in JKHS007 indicates potential emergence of an ExPEC/EAEC hybrid pathotype in wastewater.

Phenotypic and genotypic resistance. Phenotypic resistance was determined by microdilution. Nonsusceptibility/non-wild-type rates ranged from 0% (amoxicillin-clavulanic acid, piperacillin-tazobactam, imipenem, meropenem, amikacin, colistin, and fosfomycin) to 100% (ampicillin, cefazolin, cefpodoxime, cefotaxime, cefepime, and aztreonam) (see Fig. S1 in the supplemental material). The nonsusceptibile/non-wild-type rate for each antibiotic was similar between the WWTP isolates and the hospital wastewater isolates. However, we did not calculate the statistical significance because our ESBLEC collection was possibly biased due to the nonrandom selection of colonies as noted above.

Even though care should be taken in interpreting the results, the proportion of strains resistant to quinolones was quite high among our 54 ESBLEC strains (81.5% for nalidixic acid and 55.6% for ciprofloxacin according to the clinical breakpoints). Remarkably, 19 (79.2%) strains with ExPEC status were resistant to ciprofloxacin (Fig. 1), which is partly due to the predominance of strains belonging to ST131 clade C (this point is further discussed in the following section). All of the strains resistant to nalidixic acid carried at least one mutation in the quinolone resistance-determining region (QRDR), and all but two strains resistant to ciprofloxacin carried two mutations in the QRDR of *gyrA* and at least one mutation in the QRDR of *parC*, which is congruent with our previous study (20). The remaining two ciprofloxacin-resistant isolates carried one or two mutations in QRDRs in combination with quinolone resistance genes, such as *aac*(*6′*)*-Ib-cr*, *qnrS1*, *qnrS2*, *oqxA*, and *oqxB*.

All of the ESBLEC strains carried ESBL genes, and no plasmid-mediated AmpC genes or chromosomal *ampC* promoter/attenuator mutations that can result in *ampC* overexpression were detected. Among our ESBLEC strains, $bla_{\text{CTX-M-14}}$ ($n = 18$) was the most prevalent followed by $bla_{\text{CTX-M-27}}$ ($n = 15$) and $bla_{\text{CTX-M-55}}$ ($n = 9$). One isolate carried both $bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-55}}$. Importantly, 45 (83.3%) strains carried genes conferring resistance to non-β-lactam antibiotics. Cross-resistance of ESBLEC to other classes of antibiotics is of particular concern. A high level of cross-resistance in environmental ESBLEC was also observed in previous studies (21, 22). This cross-resistance may be partly due to the coexistence of ESBL genes with other resistance genes on the same plasmids (23). Detection of ESBL genes, other resistance genes, and plasmid replicons in the same contig would support this hypothesis, but this was hampered by the short read length in the present study.

Further characterization of ST131 complex strains. ESBLEC strains belonging to ST131 and its SLV were further analyzed to determine the ST131 clade/subclade of each strain. To gain insights into the fine-scale phylogeny of ST131 complex (STC131) strains, we constructed a core parsimony tree using 15 STC131 strains detected in the present study and 61 STC131 strains analyzed in our previous study (14). Strain SE15 was also included as a reference strain for clade A. kSNP3 identified 8,897 core SNPs in 77 strains. The constructed tree is shown in Fig. 2. The tree comprises three clades (A, B, and C) and four subgroups (C0, C1, C1-M27, and C2) within clade C, findings consistent with


Strain fimH ESBL Virotype QRDR
KUN2145 H22 CTX-M-14 D3 SDSE
KFEC6 H22 CTX-M-2 D3 LDSG
KKEC3 H30 TEM-12 C SDSE
KSEC7 H30 CTX-M-14 C LNSK
KUN5823 H30 TEM-132 C LDSE
KUN5781 H30 CTX-M-27 C LNIV
Ec 24 H30 CTX-M-27 C LNIV
MRSN17749 H30 CTX-M-27 C LNIV
JSWP020 H30 CTX-M-27 C LNIV
KFEC8 H30 CTX-M-27 C LNIV
SI43 H30 CTX-M-27 C LNIV
SN37 H30 CTX-M-27 NT LNIV
SN65 H30 CTX-M-27 C LNIV
S108EC H30 CTX-M-27 C LNIV
BRG120 H30 CTX-M-27 C LNIV
KSEC29 H30 CTX-M-27 C LNIV
JSWP011 H30 CTX-M-27 NT LNIV
JSWP017 H30 CTX-M-27 C LNIV
EcAZ 156 H30 CTX-M-27 C LNIV
JKHS012 H30 CTX-M-27 C LNIV
JSWP007 H30* CTX-M-27 C LNIV
JSWP014 H30 CTX-M-27 C LNIV
JSWP026 H30 CTX-M-27 C LNIV
KN1 H30 CTX-M-27 C LNIV
ONEC27 H30 CTX-M-27 C LNIV
KS26 H30 CTX-M-27 C LNIV
JSWP029 H30 CTX-M-27 C LNIV
JSWP008 H30 CTX-M-27 C LNIV
IEH71520 H30 CTX-M-27 C LNIV
KUN3594 H30 CTX-M-27 C LNIV
KT10 H30 CTX-M-27 NT LNIV
S107EC H30 CTX-M-27 C LNIV
KUN8768 H30 CTX-M-27 C LNIV
EcSA01 H30 CTX-M-14 E LNIV
ECNZ 35 H30 CTX-M-14 E LNIV
Ec 32 H30 CTX-M-14 E LNIV
FR 11 H30 CTX-M-14 C LNIV
S135EC H30 CTX-M-14 C LNIV
KT37 H30 CTX-M-14 C LNIV
Ec# 584 H30 CTX-M-27 C LNIV
USA 14 H30 CTX-M-14 C LNIV
S100EC H30 CTX-M-27 C LNIV
ONEC7 H30 CTX-M-14 C LNIV
KN94 H30 CTX-M-14 C LNIV
KUN3273 H30 CTX-M-14 C LNIV
BRG62 H30 CTX-M-14 A LNIV
KS46 H30 CTX-M-14 C LNIV
KUN4389 H30 CTX-M-14 C LNIV
JSWP032 H30 CTX-M-14 C LNIV
JKHS013 H30 CTX-M-27 C LNIV
BRG151 H30 Negative C LNIV
BRG54 H30 Negative C LNIV
SNEC5 H30 CTX-M-14 C LNIV
BRG274 H30 Negative C LNIV
KCH27 H30 CTX-M-14 C LNIV
JJ1886 H30 CTX-M-15 C LNIV
BRG221 H30 CTX-M-14+15 C LNIV
KP75 H30 CTX-M-15 E LNIV
KP46 H30 CTX-M-15 E LNIV
KS121 H30 CTX-M-15 E LNIV
KUN3842 H30 CTX-M-15 C LNIV
BRG23 H30 CTX-M-15 C LNIV
KS58 H30 CTX-M-15 C LNIV
KP14 H30 CTX-M-14+15 C LNIV
KT6 H30 CTX-M-15 C LNIV
ONEC14 H30 CTX-M-15 C LNIV
ONEC29 H30 CTX-M-15 C LNIV
SI48 H30 CTX-M-15 C LNIV
EC958 H30 CTX-M-15 A LNIV
KUN5191 H30 CTX-M-15 A LNIV
Ec 58 H30 CTX-M-15 A LNIV
Ec 31 H30 CTX-M-15 A LNIV
JSWP009 H41 CTX-M-14 NT LNIE
JSWP010 H41 CTX-M-14 NT LNIE
JKHS003 H41 CTX-M-27 E LDSE
JKHS006 H41 CTX-M-14 NT LDSE
SE15 H41 Negative NT SDSE
B
C0
C1-M27
C1
C2
C
A
500 SNPs

previous studies (13, 14). Among the STC131 strains detected in the present study, four strains belonged to clade A and 11 strains belonged to clade C. Congruent with previous studies, strains belonging to clade C were characterized by the *fimH30* allele and LNIV (S83L and D87N in GyrA and S80I and E84V in ParC) genotypes in QRDRs. Importantly, 9 of 11 clade C strains were placed in the C1-M27 clade in the phylogenetic tree. Because clade A strains lack some of the genomic regions shared among clade B and clade C strains, we also constructed a core SNP-based tree by excluding clade A strains to increase the number of informative SNP sites (see Fig. S2 in the supplemental material). The strains assigned to each ST131 clade/subclade in Fig. S2 were the same as those in Fig. 2, confirming the placement of the nine strains in the C1-M27 clade. All of these strains carried the C1-M27 clade-specific prophage-like region (M27PP1) (14) and C1-M27 clade-unique SNPs that we identified in a recent study (24). Based on these results, these nine strains (eight from the WWTP and one from hospital wastewater) were defined as belonging to the C1-M27 clade. Strains belonging to subclade C2, which is largely responsible for the global pandemic of ESBL-carrying ExPEC, were not detected in the present study. Interestingly, our recent study showed that the C1-M27 clade accounted for 38.7% of Japanese ESBL-producing ST131 isolates and was the most prevalent ST131 clade among these isolates (24). It should be noted that this clade is also present among clinical ST131 isolates from Thailand, Australia, Canada, and the United States (14), and a recent study also showed the prevalence of the C1-M27 clade in the fecal carriage of children in France (25), indicating contribution of this clade to the global spread of ST131. Although our ESBLEC collection may not represent the actual clonal compositions of ESBLEC in wastewater, the prevalence of this clade among the collection is a concern. As is the case with most clinical C1-M27 isolates, all of the C1-M27 isolates detected in the present study carried $bla_{\text{CTX-M-27}}$, and eight of these isolates were typed as virotype C. We further investigated the presence of ExPEC-associated virulence genes in clinical and environmental C1-M27 strains (see Fig. S3 in the supplemental material). Results indicate that the environmental C1-M27 strains carry similar (or even the same) sets of virulence genes compared to clinical C1-M27 strains, although the environmental strains tended to lack certain virulence genes, such as *papB*. Further studies are needed to assess the virulence potential of environmental C1-M27 strains.

In conclusion, genomic analysis of ESBLEC strains from the WWTP and hospital wastewater revealed the presence of clinically important clones among ESBLEC strains. The apparent high prevalence of fluoroquinolone resistance among these ESBLEC strains is of great concern. Core SNP-based phylogenetic analysis revealed the presence of the C1-M27 clade among our STC131 strains. This study highlights the need to monitor for antibiotic-resistant bacteria in wastewater.

MATERIALS AND METHODS

Sample collection and isolation of ESBLEC. The municipal WWTP and hospital from which the wastewater samples were collected are in the Kansai region of Japan. In October 2015, 10 samples per location were collected on different occasions from WWTP and hospital wastewater. Samples from the WWTP were collected from effluent from the final settling tanks after biological (activated sludge) treatment. Samples from the hospital consisted of untreated wastewater. A detailed description of the sampling procedure is provided in the supplemental material. Samples were processed using the membrane filter method with XM-G agar (Nissui, Tokyo, Japan) for enumeration of total *E. coli* and with chromID ESBL agar (bioMérieux, Lyon, France) for enumeration and isolation of ESBLEC. For each sample, up to four colonies showing an *E. coli* profile on the chromID ESBL plate were selected (care was taken to select colonies showing different morphologies if possible). In total, 32 and 29 isolates were obtained from the WWTP and hospital wastewater, respectively. The isolates were stored at −85°C in 35% glycerol.

Species identification and antibiotic susceptibility testing. Species identification was performed with the matrix-assisted laser desorption ionization (MALDI) Biotyper Compass 4.1 (Bruker Daltonics

FIG 2 Core SNP-based phylogenetic tree of STC131 strains. Colors of the strain names reflect the isolation sources, i.e., blue for the WWTP, red for hospital wastewater, and black for those analyzed in our previous study and SE15. Most strains in black were clinical isolates. IEH71520 was obtained from vacuum cleaner dust collected from the home of a case patient (50). SE15 was obtained from the feces of a healthy adult (51). S100EC was obtained from a rectal swab in a prospective study of returned travelers (12, 52). The QRDR column indicates amino acids of GyrA codons 83 and 87 and ParC codons 80 and 84. The ST131 clades/subclades are shown in different colors. JSWP007 carried a *fimH* allele that is different from *fimH30* by one nucleotide. NT, nontypeable.

GmbH, Bremen, Germany). Antibiotic susceptibility testing was performed by the microdilution method using the dry plate Eiken assay (Eiken, Tokyo, Japan) according to CLSI guidelines (26). The susceptibility testing included the following 25 antibiotics: ampicillin, amoxicillin-clavulanic acid, ampicillin-sulbactam, piperacillin-tazobactam, cefazolin, cefpodoxime, cefotaxime, ceftazidime, cefepime, cefoxitin, imipenem, meropenem, aztreonam, nalidixic acid, ciprofloxacin, gentamicin, tobramycin, amikacin, kanamycin, trimethoprim-sulfamethoxazole, tetracycline, minocycline, chloramphenicol, colistin, and fosfomycin. Results were interpreted according to the current epidemiological cutoff (ECOFF) values (http://mic.eucast.org/Eucast2/) as well as 2015 CLSI criteria (26). Intermediate susceptibility to each antibiotic was considered to be resistance. ESBL production was confirmed following the CLSI guidelines (26). We used cefotaxime, ceftazidime, and cefpodoxime disks with and without clavulanate (Eiken) for the confirmatory test (27).

Genome sequencing and assembly. DNA was extracted from each isolate using a DNeasy blood and tissue kit (Qiagen, Hilden, Germany), and sequencing libraries were prepared using a Nextera XT DNA sample preparation kit (Illumina, San Diego, CA). Each library was sequenced on an Illumina MiSeq instrument for 600 cycles (300-bp paired-end reads) to achieve an average sequencing depth of 80. Raw reads from each sample were trimmed using ERNE-FILTER (28) and assembled using SPAdes v3.10.0 (29). Assemblies were improved using Pilon (30).

Genomic analysis. Assembled contigs were subjected to the following analysis. Acquired resistance genes were detected using the ResFinder antimicrobial resistance gene database (31) and the ARG-ANNOT database (32). Chromosomal *ampC* promoter/attenuator mutations that can result in *ampC* overexpression were analyzed as previously described (33). Mutations in quinolone resistance-determining regions (QRDRs) of *gyrA* and *parC* were analyzed according to Aoike et al. (34). Plasmid replicons were detected using PlasmidFinder (35). MLST *in silico* was performed using the MLST web tool (36) and EnteroBase (http://enterobase.warwick.ac.uk). Phylogenetic groups were determined as described previously (20). The *fimH* allele types, which enable further discrimination of strains belonging to the same ST, were determined according to the classification system established previously (37). Parsimony trees based on SNPs in whole-genome data were constructed using kSNP3 (38, 39). Virulence genes were detected using an in-house database compiled from the Virulence Factors Database (VFDB) (40) and by literature review (41–47). *E. coli* pathotypes were defined based on the presence of specific virulence genes (48). ST131 virotypes were determined as described previously (49).

Accession number(s). Sequence data obtained in the present study have been deposited in the DDBJ Sequence Read Archive database under DDBJ accession number DRA005619.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.00564-17.

SUPPLEMENTAL FILE 1, PDF file, 0.7 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.1 MB.

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