Global *Escherichia coli* Sequence Type 131 Clade with *bla*<sub>CTX-M-27</sub> Gene


The *Escherichia coli* sequence type (ST) 131 C2/H30Rx clade with the *bla*<sub>CTX-M-15</sub> gene had been most responsible for the global dissemination of extended-spectrum β-lactamase (ESBL)–producing *E. coli*. ST131 C1/H30R with *bla*<sub>CTX-M-27</sub> emerged as an important ESBL-producing *E. coli* in Japan during the late 2000s. To investigate the possible expansion of a single clade, we performed whole-genome sequencing for 43 Japan and 10 global ST131 isolates with *bla*<sub>CTX-M-27</sub> (n = 16), *bla*<sub>CTX-M-14</sub> (n = 16), *bla*<sub>CTX-M-15</sub> (n = 13), and others (n = 8). We also included 8 ST131 genomes available in public databases. Core genome-based analysis of 61 isolates showed that ST131 with *bla*<sub>CTX-M-27</sub> from 5 countries formed a distinct cluster within the C1/H30R clade, named C1-M27 clade. Accessory genome analysis identified a unique prophage-like region, supporting C1-M27 as a distinct clade. Our findings indicate that the increase of ESBL-producing *E. coli* in Japan is due mainly to emergence of the C1-M27 clade.

The global increase in resistance to the third-generation cephalosporins and fluoroquinolones among extraintestinal pathogenic *Escherichia coli* (ExPEC) is a public health concern because of the importance of these drugs in treating serious infections (1). The extended-spectrum β-lactamases (ESBLs), especially CTX-M types, contribute to third-generation cephalosporin resistance among ExPEC, and specific mutations in quinolone resistance–determining regions in *gyrA* and *parC* mainly contribute to fluoroquinolone resistance (2). The increase in resistance among ExPEC has resulted mainly from the recent expansion of a pandemic clonal group known as *E. coli* sequence type (ST) 131, which is usually multidrug resistant and is associated with CTX-M-15, the most prevalent β-lactamase among ESBL-producing ExPEC (2). ST131 harbors more virulence factors than other antimicrobial-resistant ExPEC and can cause severe infections (2,3).

Recent studies using whole-genome sequencing (WGS) analysis revealed that ST131 comprises different lineages or clades (4,5). Price et al. found a dominant fluoroquinolone-resistant lineage (named H30R) in North America that contains the *fimH* 30 allele and was associated with characteristic quinolone resistance–determining region mutations (2,4). ST131 with the *bla*<sub>CTX-M-15</sub> gene formed a distinct cluster within the H30R lineage, referred to as the H30Rx clade (4). Petty et al. confirmed these findings using a collection of strains from 6 countries (5). In their study, H30R and H30Rx clades correspond to clade C and clade C2 (subset of clade C), respectively. The other clade C subset, clade C1, included ST131 isolates with different CTX-Ms than *bla*<sub>CTX-M-15</sub>.

Globally, the CTX-M-15–producing C2/H30Rx clade is mostly responsible for the pandemic of ExPEC with ESBLs (2), but in Japan, ExPEC with *bla*<sub>CTX-M-15</sub> is rare despite the predominance of ST131 among ESBL-producing isolates (6). Before 2005, ST131 C1/H30R negative for Rx containing *bla*<sub>CTX-M-14</sub> predominated among Japanese ST131 (6). In 2006, ST131 C1/H30R with *bla*<sub>CTX-M-27</sub> was detected in Japan, and the numbers of this lineage escalated since 2010 and are responsible for the substantial increase in ESBL-producing ExPEC in Japan (6). Moreover, *bla*<sub>CTX-M-27</sub> is confined to ST131, whereas other CTX-Ms, such as *bla*<sub>CTX-M-14</sub> and *bla*<sub>CTX-M-15</sub>, are equally present among ST131 and non-ST131 *E. coli* isolates (3).

increase of CTX-M-27–producing ST131 in Japan since 2010 (6), we designed a study to characterize these isolates using WGS techniques.

Materials and Methods

Bacterial Isolates
We selected 43 nonduplicate ST131 clinical isolates collected from 2 multicenter surveillance programs in Japan for WGS to represent 3 major ESBL-producing ST131 (CTX-M-27–producing H30R, 13 isolates; CTX-M-14–producing H30R, 9 isolates; CTX-M-15–producing H30Rx, 11 isolates) and other ST131 (CTX-M-14+CTX-M-15–producing H30Rx, 2 isolates; CTX-M-14–producing H30Rx, 1 isolate; CTX-M-14–producing H22, 1 isolate; CTX-M-2–producing H22, 1 isolate; TEM-producing H30, 2 isolates; non–ESBL-producing H30R, 3 isolates) in Japan (6) (Table). One of the surveillance programs collected ESBL-producing E. coli isolates during 2001–2010 at 10 acute-care hospitals in the Kyoto and Shiga prefectures of Japan (6); the other program collected all E. coli isolates during December 2014 at 10 acute-care hospitals in the 5 prefectures in central Japan. ST131 isolates were identified by PCR specific for mdh and gyrB alleles, O25b or O16 rfb variants, fimH allele, and H30Rx status (6). The selection process of the Japanese ST131 ensured equal representation by geographic location, specimen type, and date of isolation.

In addition to isolates from Japan, we obtained 10 CTX-M–producing ST131 isolates from global collections that previously had been characterized by multilocus sequence typing (MLST) (Table; online Technical Appendix Table 1, http://wwwnc.cdc.gov/EID/article/22/11/16-0519-Techapp1.pdf). We selected all of the CTX-M-27 producers, 1 CTX-M-14 producer per country, and 2 CTX-M-15 producers. We also sought public databases for ST131 H30 and included sequence data for 8 isolates from countries other than Japan: CTX-M-27 producers (3 raw reads, 2 draft genomes); CTX-M-14 producer (1 raw read); and CTX-M-15–producing C2/H30Rx (2 complete genomes) (Table; online Technical Appendix Table 1) (5,9,16–18).

WGS
We used the Nextera XT DNA sample preparation kit (Illumina, San Diego, CA, USA) to prepare libraries for sequencing. Samples were multiplexed and sequenced on an Illumina MiSeq for 600 cycles (300-bp paired-end) or NextSeq500 for 300 cycles (151-bp paired-end). The ST131 genomes were sequenced at an average depth of 44.03 (SD ± 14.70) and an average coverage of 97.33% (SD ± 0.93%) using the 5,109,767-bp EC958 chromosome as previously described (16).

Core Genome Analysis
We used a core genome single-nucleotide polymorphism (SNP)–based approach to create a phylogenetic tree. We identified SNPs using raw read mapping followed by duplicate read removal, realignment, quality score recalibration, and variant filtering (online Technical Appendix). Reads from 53 isolates sequenced in this study and 4 isolates (S100EC, S107EC, S108EC, and S135EC) (5) were aligned against a reference genome of EC958, and SNPs were called. The remaining 4 draft or complete genomes underwent whole-genome alignment against EC958 to make EC958-like pseudo-chromosomes that contained only SNPs. The SNP-only core genome was identified as the blocks of >500 bp common to all 61 study isolates to ensure that each block represented a common segment from good alignment in each isolate and that the block had enough length to enable identification (5). A maximum-likelihood tree was built using RAxML (19). A recombination-free tree was also built by excluding recombination sites identified using a Bayesian analysis software BRATNextGen (20).

Comparative Genomic Analysis
To define presence of genes and their alleles, we used SRST2 with trimmed reads or BLAST+ (executables [http://blast.ncbi.nlm.nih.gov/]) with assembled draft genomes and following databases or typing schemes: ResFinder antimicrobial resistance gene database, VFDB and VirulenceFinder virulence gene databases, serotypeFinder O:H typing database, PlasmidFinder plasmid database, MLST (http://mlst.ucc.ie/mlst/dbs/Ecoli), plasmid MLST, fimH typing, gyrA(parC) typing, ST131 virotyping, and detection of H30Rx–specific ybbW SNPs, plasmid addiction systems, and blaCTX-M genetic environment (online Technical Appendix). We used pangenome analysis to identify clade specific segments among draft or complete genomes. BRIG was used to visualize similarity of genomes to ST131 genomic islands (16) and to the ST131 reference plasmid pEC958 (21).

Statistical Analysis and Sequence Data

Accession Numbers
We compared categorical variables using Fisher exact test. A p value <0.05 was considered statistically significant. We conducted our statistical analysis using Stata, version 13.1 (StataCorp, College Station, TX, USA). The sequences were deposited in the DDBJ Sequence Read Archive database (accession no. DRA004266 and DRA004267).

Results

Bacterial Isolates
The study comprised 60 clinical and 1 environmental ST131 isolates (Table; online Technical Appendix Table
1). We confirmed the types of β-lactamase genes, ST131 status, 
*fimH* allele numbers, and *H30Rx* status using draft genomes.

### Core Genome SNP-based Phylogenetic Tree

We identified a 4,086,650-bp core genome that included 5,280 SNPs by mapping and alignment of the 61 study isolates to EC958 (Figure 1). The ciprofloxacin-resistant isolates with *gyrA* 1AB and *parC* 1aAB alleles formed the C/H30R cluster that comprised the C2/H30Rx and C1/ 

The C1-M27 Clade—Specific Region

The panengenome analysis of genomes from all the isolates identified an 11,894-bp region named M27PP1 that was specific to all the isolates from the C1-M27 clade. Further analysis using the BLAST database and Sanger sequencing for gap filling showed that this region was identical to a prophage-like genomic island (GenBank accession no. CP006632) in *E. coli* isolate in the United States. M27PP1 was inserted into the core genome–based phylogeny with or without recombination sites showed the same results as the phylogenetic tree obtained with recombination sites (online Technical Appendix Figure 2). In addition to the core–genome–based phylogeny with or without recombination sites, the C1-M27 clade was defined by a unique accessory genome of the M27PP1.
whereas the 2 plasmids (pEQ011, pSD853_89) contained only a 44-bp similar segment at 5’ side and other parts of these plasmids were not found in the C1-M27 clade isolates.

Two *E. coli* ST131 C1-M27 isolates (i.e., KUN5781 and Ec 24) had an additional insertion region of 19,352 bp, named M27PP2, situated upstream of M27PP1. M27PP2 was accommodated within the same 7-bp direct repeat region (Figure 2). M27PP2 included a 15,552-bp region that showed 88.9% homology to a prophage-like sequence in the chromosome of the γ proteobacterium Hdn1 (GenBank...

**Figure 1.** Core genome single-nucleotide polymorphism (SNP)–based phylogenetic tree of *Escherichia coli* sequence type (ST) 131 isolates. This maximum-likelihood phylogram is based on a 4,086,650-bp core genome and a total of 5,280 SNPs. The tree is rooted by using the outgroup *H*22 isolates, and asterisks indicate bootstrap support >90% from 100 replicates. Strains that had previously been sequenced are in italics. The Country columns indicate places of isolation: Ja to Jw, Japan (a to w indicates hospitals); AU, Australia; CA Canada; FR, France; NZ, New Zealand; SA, South Africa; TH, Thailand; UK, United Kingdom; US, United States; VI, Vietnam. Environment column shows a type of genetic environment of ESBL genes (online Technical Appendix Table 2, http://wwwnc.cdc.gov/EID/article/22/11/16-0519-Techapp1.pdf). FQ columns indicate ciprofloxacin susceptibilities (S, susceptible; R, resistant). KSEC7 had a parC 1aE allele including G250A (S80K) mutation in addition to a 1a allele. The ciprofloxacin-resistant C/H30Rx cluster comprised the C2/H30Rx and C1/H30R clades. All of the H30Rx isolates belonged to the C2/H30Rx clade. The C1/H30R clade included CTX-M-14–producing H30R, non–ESBL-producing H30R, and CTX-M-27–producing H30R isolates. CTX-M-27–producing isolates belonged to the C1-M27 clade within the C1/H30R clade except 2 isolates (S100EC and Ec# 584). The bootstrap value for the root of the C1-M27 clade was 64%. An average of 68 SNPs was found among the C1-M27 clade, whereas an average of 158 SNPs was found between the C1-M27 clade and 2 non–C1-M27 clade isolates with btaCTX-M-27. Scale bar indicates 100 SNPs.
accession no. FP929140) and 99.8% homology to the insertion element ISSen4.

Genomic Comparison of the ST131 Genomic Islands and Virulence Genes

The sequences of the study isolates were similar to the ST131 genomic islands in EC958 and JJ1886 (a CTX-M-15–producing C2/H30Rx strain obtained in the United States from a patient with fatal urosepsis) (Figure 3) (17). The C1-M27 clade isolates lacked the prophage 1 region present in EC958 (Figure 3). This prophage 1 region, specific for ST131, was present among the non–C1-M27 ST131 isolates in this study, except for BRG23 and EcSA01. The presence of ExPEC-associated virulence genes is shown in online Technical Appendix Figure 3.

Plasmid Replicons, Addiction Systems, and Antimicrobial Drug Resistance Genes

We compared the study isolates with pEC958, the plasmid present in EC958 that carries blaCTX-M-15 (online Technical Appendix Figure 4). The C1-M27 clade lacked the first part of the transfer region (tra) present in pEC958. Some regions common to both C2/H30Rx and C1/H30R clades were present in pEC958. The C1/H30R clade producing CTX-M-27 or CTX-M-14 (including C1-M27) contained mostly F1:A2:B20 replicons, whereas the C2/H30Rx clade producing CTX-M-15 contained mainly F2:A1:B- replicons (online Technical Appendix Figure 5). The C1-M27 clade isolates were negative for Tn2 containing blaTEM-1. Two C1-M27 isolates from Thailand and the United States were also positive for blaNDM-1 (online Technical Appendix Figure 5).

Discussion

A previously unreported clade named C1-M27 within C1/H30R clade is responsible for the epidemic of ESBL-producing ExPEC in Japan and has already been disseminated to 5 countries on 3 continents. ST131 containing blaCTX-M-27 is responsible for human infections has been reported from various continents (2) and is especially common among ESBL-producing ExPEC in certain countries, such as Israel, the Czech Republic, and Switzerland (2,13,14). CTX-M-27–producing ST131 also is present among nonclinical and nonhuman E. coli isolates, including fecal specimens of healthy children attending day care centers in France; fecal specimens of healthy adults in China, Portugal, and the Netherlands; samples from sick dogs and cats in Japan; samples from water birds from central Europe and Swiss rivers and lakes; and samples of well water from China (2,10,11,15,23–25). The most common ESBL among E. coli ST131 in nonhuman samples is CTX-M-27 (2,23–25). ST131 with blaCTX-M-15 is rare among animal and environmental E. coli isolates (26). Our analysis of IEH71520,
**Figure 3.** Genome similarities to the *Escherichia coli* sequence type (ST) 131 genomic islands and the C1-M27 clade–specific region. Rings drawn by BRIG show the presence of these regions. Colored segments indicate >90% similarity and gray segments indicate >70% similarity by BLAST comparison between the regions of interest and each genome. Extended-spectrum β-lactamase types are indicated in parentheses of Type column. The regions from Flag2 to GI-leuX were found in EC958, the prophage 8 region was found in JJ1886, and the M27PP1 and M27PP2 were found as the C1-M27 clade–specific regions in this study. Prophage 6, capsule, GI塞尔C, and prophage 8 regions were present in some C2/H30Rx isolates but were absent in C1/H30R isolates.

Recent studies focusing on evolutionary history of ST131 suggested that C1/H30R and C2/H30Rx clades emerged ≈30 years ago, after their acquisition of gyrA-1AB and parC-1aAB alleles from C0/H30 (non-R) clade (27,28). The phylogeny and smaller numbers of SNPs in the C1-M27 clade (Figure 1) suggest that this clade was recently diverged from the C1/H30R. In the time-scaled phylogeny presented by Stoesser et al. (27), a cluster that included 6 CTX-M-27–producing isolates from Cambodia, Thailand, and Laos in 2007–2011 was present within the C1/H30R clade. This cluster, supposed to be the C1-M27 clade, diverged in the early 2000s, supporting our hypothesis.

CTX-M-27–producing ST131 that belongs to the H41 lineage previously had been described from Japan (6) and China (13). The characterization of the Japanese ST131
$H41$ isolates showed different genetic structures flanking the $\text{bla}_{\text{CTX-M-27}}$, from those structures present in $E. coli$ ST131 $H30R$ ($6$). The flanking regions previously characterized in ST131 $H41$ were identical to the flanking regions in ST131 non–C1-M27 from this study. It seems there are $2$ types of structures flanking the $\text{bla}_{\text{CTX-M-27}}$ among $E. coli$ ST131; $1$ type is confined to clade C1-M27 (i.e., $208$ bp of $\Delta\text{IS}Ecp1$ upstream and $\text{IS}903D$ downstream), whereas another structure (i.e., $388$ bp of $\Delta\text{IS}Ecp1$ upstream and full $\text{IS}903D$ downstream) is distributed among non–C1-M27 isolates, including ST131 $H41$ ($6$). Therefore, ST131 $H41$, through horizontal transfer of $\text{bla}_{\text{CTX-M-27}}$, is unlikely to have played a substantial role in the emergence of the C1-M27 clade.

Two ST131 isolates with $\text{bla}_{\text{CTX-M-27}}$ from Australia and Vietnam did not belong to the C1-M27 clade (Figure 1). These $2$ isolates differ from the C1-M27 clade in that their core genomes had more SNPs (158 vs. 68), contained the prophage $1$ ST131-specific region, and lacked the M27PP1 and M27PP2 elements. Moreover, the genetic environment surrounding the $\text{bla}_{\text{CTX-M-27}}$ differed from $E. coli$ ST131 C1-M27 (as described previously). The isolate from Vietnam lacked $\text{mph}(A)-\text{mrx}-\text{mphR}$, tetR-tet(A), $\text{su}l2-strA-strB$, and $\text{In}54$ resistance genes, compared with the C1-M27 clade (online Technical Appendix Figure $5$). These differences indicate that some ST131 isolates might acquire $\text{bla}_{\text{CTX-M-27}}$ independently from the C1-M27 clade.

Our study has several limitations. Most isolates originated from Japan. However, we were able to include ST131 C1-M27 isolates from $5$ countries on $3$ continents and C1/H30R isolates producing CTX-M-14 or CTX-M-15 from $6$ countries on $4$ continents. Another limitation was that we were able to obtain only $1$ environmental ST131 isolate with $\text{bla}_{\text{CTX-M-27}}$ (IEH71520). Future studies that include environmental isolates will provide additional insights into molecular epidemiology and evolutionary history of the C1-M27 clade. We could not analyze plasmid contents of $\text{bla}_{\text{CTX-M-27}}$ because $\text{bla}_{\text{CTX-M-27}}$-containing contigs were too short. The sequencing of plasmids that contain $\text{bla}_{\text{CTX-M-27}}$ obtained from various ST131 clades (including the C1-M27 clade) should also be undertaken.

In conclusion, we showed that the recent increase in ESBL-producing $E. coli$ from Japan resulted from emergence of a ST131 C1/H30R subclade with $\text{bla}_{\text{CTX-M-27}}$. This clade, named C1-M27, had unique genomic characteristics and was present in ST131 from Thailand, Australia, Canada, and the United States. Our findings suggest that the C1-M27 clade is contributing to the global success of ST131. $E. coli$ ST131 C1-M27 poses a major new public health threat because of its global distribution and association with the very dominant C/H30 lineage. We urgently need rapid cost-effective detection methods for $E. coli$ ST131 C1-M27 and well-designed epidemiologic and molecular studies to understand the dynamics of transmission, risk factors, and reservoirs for ST131 C1-M27. These efforts will provide insight into the emergence and spread of this multidrug-resistant clade that will lead to information essential for preventing the spread of ST131.

Acknowledgments
We thank the members of the Kyoto-Shiga Clinical Microbiology Study Group (Naohisa Fujita, Toshiaki Komori, Yukiji Yamada, Tsuneo Shimizu, Akihiko Hayashi, Tamotsu Ono, Harumi Watanabe, Naoko Fujihara, Takeshi Higuchi, Kunihiko Moro, Masayo Shigeta, Kaneyuki Kida, Hiromi Terada, Fusayuki Tsuboi, Yoshishia Sugimoto, and Chiyoko Fukumoto) and the members of the 89JAID–BRG (89th Annual Meeting of the Japanese Association for Infectious Diseases–drug-resistant Bacteria Research Group; Ooi Yukimasa, Tomohiro Higashiyama, Masaaki Sasano, Harumi Watanabe, Tsunehiro Shimizu, Akihiko Hayashi, Yasufumi Matsumura, Taro Noguchi, Miki Nagao, Masaki Yamamoto, Michio Tanaka, Takeshi Higuchi, Toru Kanahashi, Naohisa Fujita, Toshiaki Komori, Yukiji Yamada, Kohei Ueda, Go Yamamoto, Kunihiko Moro, and Shigenobu Yabu) for collection of isolates. We also thank Sayo Shitashiro and Karen Poon for technical assistance.

This work was supported by Japan Society for the Promotion of Science (JSPS) Grant-in-Aid for Young Scientists (B) Grant No. 15K19179 (Y.M.), the John Mung Program from Kyoto University, Japan (Y.M.), and a research grant from the Calgary Laboratory Services (#10006465; J.D.D.P.). J.D.D.P had previously received research funds from Merck and Astra Zeneca.

Dr. Matsumura is an assistant professor at the Clinical Laboratory Medicine, Kyoto University Graduate School of Medicine, Kyoto, Japan. His main research focuses on the detection and molecular epidemiology of antimicrobial drug resistance mechanisms among gram-negative bacteria.

References


Address for correspondence: Yasufumi Matsunami, Department of Clinical Laboratory Medicine, Kyoto University Graduate School of Medicine, 54 Shogoin Kawahara-cho, Sakyo-ku, Kyoto 6068507, Japan; email: yazblood@kuhp.kyoto-u.ac.jp