Dissemination of IncF group F1:A2:B20 plasmid-harboring multidrug-resistant *Escherichia coli* ST131 before the acquisition of *bla*$_{CTX-M}$ in Japan

bla$_{CTX-M}$の獲得以前のIncFグループF1:A2:B20プラスミドを保有する多剤耐性大腸菌ST131の日本における広まり

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ABSTRACT

Objectives: The *Escherichia coli* O25-ST131 clone is responsible for global dissemination of *bla*$_{CTX-M}$. However, the prevalence of this clone in the digestive tract, devoid of antimicrobial selection, and its molecular epidemiology remain unclear. In this study, we examined the origin of *bla*$_{CTX-M}$-positive *E. coli* O25-ST131 and its distribution.

Methods: We separately sequenced the chromosomal and plasmid genomes of 50 *E. coli* O25 isolates obtained from fecal samples of patients with diarrhea in Japan.

Results: Although 36 of 50 (72%) *E. coli* O25 isolates were ST131, only 6 harbored *bla*$_{CTX-M}$. According to the *fimH* and *ybbW* sequences and fluoroquinolone susceptibility, *H*30R1 isolates were dominant (27/36; 75%) and possessed IncFII- FIA-FIB with FAB formula subtype F1:A2:B20 plasmids at a high frequency (24/27; 89%). The F1:A2:B20 plasmids possessed more resistance genes such as *bla*TEM-1, aminoglycoside resistance genes, and sulfamethoxazole-trimethoprim resistance genes compared to non-F1:A2:B20 plasmids. In contrast, only one *bla*$_{CTX-M-14}$ was located on the F1:A2:B20 plasmids, whereas the other three were located on IncFII (F4:A-:B-) (n = 1) and IncZ plasmids (n = 2). Two *H*30Rx-ST131 isolates harbored *bla*$_{CTX-M-15}$: one was on the chromosome and the other on the IncFIA-R plasmid. The

stability and conjugation ability of the F1:A2:B20 plasmids were compared with those of non-F1:A2:B20 plasmids, which revealed higher stability but were less conjugative ability.

Conclusions: These results suggest that *E. coli H*30R1-ST131 is a multidrug-resistant clone containing several resistance genes in the F1:A2:B20 plasmid, which were widely distributed before the acquisition of $bla_{CTX\text{-}M}$.

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目的：大腸菌O25-ST131は*bla*$_{CTX-M}$遺伝子の世界的拡散を担っているクローン株である。しかし、抗菌薬選択下にない腸管内におけるこのクローンの分布や、その分子疫学的特徴は未だ明らかになっていない。本研究では、 *bla*$_{CTX-M}$ 陽性大腸菌 O25-ST131 の起源やその分布について検証した。

方法：下痢を発症した日本人患者の糞便から分離された血清型O25の大腸菌50株の染色体ゲノムとプラスミドゲノムを別々に解析した。

結果：血清型O25の大腸菌50株の内、36株がST131であったが、*bla*$_{CTX-M}$を保有していたのは6株だけであった。*fimH*と*ybbW*の塩基配列およびキノロン感受性結果に基づいて、分類したところ、*H*30R1 株が最も優勢で (27/36; 75%) 、それらは高い頻度でF1:A2:B20というサブタイプの IncFII-FIA-FIB プラスミドを保有していた (24/27; 89%)。そのF1:A2:B20プラスミドはnon-F1:A2:B20と比較して、より多くの耐性遺伝子を持っており、例を挙げると*bla*TEM-1、アミノグリコシド耐性遺伝子、トリメトプリム/スルファメトキサゾール耐性遺伝子であった。しかし、F1:A2:B20上に存在する*bla*$_{CTX-M-14}$遺伝子は1つだけで、それ以外の*bla*$_{CTX-M-14}$遺伝子3つはIncFII (F4:A-:B-) (n = 1)とIncZ (n = 2) プラスミドに位置していた。2つの*H*30Rx-ST131株は*bla*$_{CTX-M-15}$を保有し、一つは染色体上に、もう一つはIncFIA-Rプラスミド上にあった。 また、

F1:A2:B20プラスミドはnon-F1:A2:B20プラスミドと比較して、高い安定性と低い接合効率を示した。

結論：これらの結果は、F1:A2:B20プラスミドが*bla*$_{CTX-M}$を獲得する前に広まったプラスミドであり、大腸菌*H*30R1-ST131が、これらのプラスミド上にいくつもの耐性遺伝子を持つ多剤耐性クローン株であることを明らかにした。

【CONTENTS】

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Running title: Dissemination of MDR ST131 clones with F1:A2:B20

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Keywords: *Escherichia coli* ST131, CTX-M type ESBL, digestive tract colonization, Plasmid analysis, IncF F1:A2:B20

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Conclusions: These results suggest that *E. coli H*30R1-ST131 is a multidrug-resistant clone containing several resistance genes in the F1:A2:B20 plasmid, which were widely distributed before the acquisition of bla_{CTX-M}.

1 INTRODUCTION

In the past two decades, prevalence of CTX-M-type extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* has been increasing worldwide (1). Global dissemination of the high-risk clone *E. coli* O25-ST131, which is multidrug-resistant, poses a crucial threat to public health and this clone frequently produces CTX-M type β-lactamase (2). Although the prevalence of *E. coli* ST131 has been widely reported among ESBL-producing or fluoroquinolone-resistant isolates, few studies have investigated the prevalence of *E. coli* ST131 among all *E. coli* isolates regardless of antimicrobial resistance. Furthermore, the digestive tract carriage of this high-risk *E. coli* ST131 clone in the absence of antimicrobial selection has not been widely examined (3-6).

Presence of *E. coli* ST131 in the digestive tract may also be investigated using fecal samples obtained from diarrhea patients. BML, Inc., one of the largest Japanese commercial laboratories, reported the O-serotype prevalence of *E. coli* isolates in diarrheal stools on their website (http://www.bml.co.jp/bct_info/c/). The 15-year trends (1999–2013) observed for four major O serotypes found in Japan are shown in Fig. S1. Notably, the proportion of O25, which was only 3% in 1999, increased to 11% in 2002 and reached 22% in 2013. Because *E. coli* ST131 consists mostly of serotype O25:H4 with some exceptions (7), we hypothesized that most *E. coli* O25 detected in patients with diarrhea were ST131, and the increase in O25 among *E. coli* from stool samples occurred because of increased colonization of the digestive tract by *E. coli* ST131 in the absence of antimicrobial pressure.

Application of recently developed sequencing techniques enabled detailed analysis of the molecular epidemiology of *E. coli* ST131. *H*30Rx and *H*30R1 (also known as clades C2 and C1, respectively) subclonal groups, which were most prevalent within CTX-M-type ESBL-producing *E. coli* ST131, were found to be associated with $bla_{\text{CTX-M-15}}$ and $bla_{\text{CTX-M-14}}$/$bla_{\text{CTX-M-27}}$,

respectively (8-13). The *H*30Rx subclonal group with $bla_{CTX\text{-}M\text{-}15}$ has been identified globally, particularly in Europe and the United States, whereas the *H*30R1 subclonal group with $bla_{CTX\text{-}M\text{-}14}$ or $bla_{CTX\text{-}M\text{-}27}$ was predominantly found in East Asia (11,12,14). An emerging clone, C1-M27, was recently reported to be responsible for the CTX-M-27-producing *E. coli* epidemic in Japan (15). The association between IncF plasmids and the ST131 subclones has been also studied, and the *H*30Rx and *H*30R1 subclonal groups were found to harbor plasmids with replicons of IncFII-FIA and IncFII-FIA-FIB with FAB formulae F2:A1:B- and F1:A2:B20, respectively (13,15,16). However, most of these findings were based on isolates selected via ESBL production. It is important to clarify whether clones such as *H*30Rx-ST131 and *H*30R1-ST131 and their associated IncF group plasmids were disseminated as a reservoir before the acquisition of $bla_{CTX\text{-}M}$.

Utilizing recently developed sequencing technologies, we investigated the detailed molecular epidemiology of our historical *E. coli* O25 isolate collection isolated in 2008 from fecal samples of Japanese diarrhea patients to assess the role of this clone in $bla_{CTX\text{-}M}$ dissemination.

2 MATERIALS AND METHODS

2.1 Bacterial isolates and antimicrobial susceptibility testing

Fifty *E. coli* O25 isolates recovered from the diarrheal stools of 50 patients between October and December 2008 were obtained from a commercial clinical laboratory in Japan. Those stools were submitted to the laboratory from different clinics and hospitals across Japan. Antimicrobial susceptibility testing was performed using a MicroScan Walkaway system (Beckman Coulter,US). The results were interpreted using 2016 Clinical and Laboratory Standards Institute breakpoints (17).

2.2 DNA sequencing

To analyze the draft genome sequence of chromosomes and plasmids separately, S1 nuclease pulsed-field gel electrophoresis (S1-PFGE) was performed as described previously with some modifications (18), followed by whole genome sequencing. Briefly, chromosomal DNA and plasmid DNA were separated by treating DNA agarose gel plugs into 36 units of S1 nuclease (Takara Bio, Shiga, Japan) in 100 μL S1 nuclease buffer (Takara Bio) at 37°C for 40 min followed by PFGE. The DNA bands were visualized using a SYBR Gold nucleic acid gel stain (Life Technologies, Carlsbad, CA, USA) under a blue-light transilluminator. Chromosomal bands and visible plasmids bands larger than 25 kb in size were cut out from the gel and their DNA was purified. The DNA sequencing library was prepared and sequencing reactions were conducted on an Illumina MiSeq sequencer (Illumina, Inc., San Diego, CA, USA) as previously described (19).

2.3 Analysis of plasmid DNA

De novo assembly of plasmid DNA was performed using the A5-miseq pipeline (20), followed by annotation with the Prodigal program (version 2.60) (21), from which the sequence data for 114 plasmids were obtained. Thirteen plasmids were excluded from analysis because they possessed sequences identical to those of other plasmids harbored by the same hosts. Finally, 101 plasmids were included in our study. The median number of contigs per plasmid was 4, which ranged from 1 to 20. The mean coverage of plasmids varied from 26.84 to 2338.56. For most of the plasmids (80/101, 79.2%), the mean coverage was more than 100 and for 19 plasmids(19/101,18.8%) it was from 32.37 to 96.69. There were only two plasmids for which the mean coverage was less than 30(26.84 and 29.77). Plasmid replicon types were searched using Center for Genomic Epidemiology server, PlasmidFinder 1.3 (https://cge.cbs.dtu.dk/services/PlasmidFinder/)(22). IncF replicon sequence types were identified by searching for sequences homologous to the allele sequences registered in the

Plasmid PubMLST database (https://pubmlst.org/plasmid/) (23). Resistance genes were identified using the Center for Genomic Epidemiology ResFinder 2.1 (http://www.genomicepidemiology.org) (24).

2.4 Detection of ST131 and its subclonal groups

Chromosomal DNA was assembled using an A5-miseq pipeline and analyzed for sequence types (STs) and subclonal groups. Multilocus sequence typing (MLST) was performed according to the Achtman scheme using the EnteroBase ST finding tool (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli). Next, *fimH* alleles were characterized by comparing 64–552-nucleotide sequences of *fimH* (25, 26). Ciprofloxacin- susceptible and ciprofloxacin-non-susceptible *H*30 isolates were classified as *H*30S and *H*30R, respectively. The *H*30R subclonal group was examined for *H*30Rx-specific *ybbW* (allantoin transporter-encoding gene) single-nucleotide polymorphisms to identify *H*30Rx (27). In the current study, non-*H*30Rx was referred to as *H*30R1 based on the study by Johnson *et al*. (13).

2.5 DNA sequencing and analysis by PacBio RSII

Four isolates were sequenced using PacBio RSII (Pacific Biosciences, Menlo Park, CA, USA) with single-molecule real-time sequencing (SMRT) cells, according to the manufacturer's instructions. De novo assembly was performed using the PacBio SMRT Portal protocol RS_HGAP Assembly 3. To confirm the accuracy of the completed plasmid sequences, the sequences obtained via PacBio RSII were compared with those obtained via Illumina MiSeq using the Hybrid Finishing Tool for PacBio and Illumina (HyFi-PI). Plasmid replicon types and resistance genes were annotated and identified as described previously.

2.6 Plasmid transfer experiments

Conjugal transfer of plasmids from 9 donor isolates into *E. coli* DH10B harboring pHSG298 (Takara Bio, Shiga, Japan) was performed using liquid mating experiments, in which pHSG298 was transferred by electroporation to *E. coli* DH10B to confer kanamycin resistance. Donor

isolates and recipient strains were grown overnight in 3 mL of Luria-Bertani (LB) broth containing antimicrobials. On the next day, these cultures were washed to remove the antimicrobials by spinning down 1 mL of culture and resuspending the pellet in 1 mL of LB broth. Donor and recipient cells were mixed at volumes of 0.2 and 0.8 mL, respectively, and incubated at 37°C without shaking for 3 h after centrifugation at room temperature. After incubation, the cultures were serially diluted and plated in 100-μL aliquots onto Mueller-Hinton (MH) agar containing each selective antimicrobial for donor plasmid selection and 100 μg/mL kanamycin. Antimicrobials for donor plasmid selection were chosen according to the resistance genes present on the subject plasmids, such as 100 μg/mL ampicillin for *bla*TEM-1, 4 μg/mL cefotaxime for $bla_{\text{CTX-M-14}}$, 4 μg/mL trimethoprim for *dfrA24*, and 128 μg/mL sulfamethoxazole for *sul1*. To confirm the transconjugant, 10 colonies were randomly selected for analysis via PCR assays for resistance genes and possessed by donor plasmids. In addition, one colony was subjected to S1-PFGE to confirm the possession of plasmid and PFGE using XbaI (New England Biolabs, Ispwich, MA, USA) to confirm the recipient. Conjugation efficiencies were expressed as the number of transconjugants per donor cell. Experiments were performed in triplicate for each isolate combination.

2.7 Plasmid stability experiments

Plasmid stability was assessed using five transconjugants obtained from the above experiments through daily passage in LB broth with and without ciprofloxacin for 20 days as previously described (13). The concentration of ciprofloxacin was 0.001 μg/mL, which is half of the minimum inhibitory concentration for the host cells. Briefly, 3 μL overnight cultures were inoculated into 3 mL fresh LB broth and incubated for 12 h at 37°C. After every 12 hours, 3 μL full-grown culture was transferred to 3 mL fresh LB broth. On days 0, 5, and 10, samples drawn from the growth were serially diluted and plated onto MH agar without antimicrobials. Next, 100 colonies were replicated on the MH agar with and without antimicrobials for selection. PCR

assays of antimicrobial resistance genes or PCR-based replicon typing were performed on 10 colonies from each isolate and time point to confirm the presence of plasmids.

2.8 Statistical analysis

Categorical variables were compared using Fisher's exact test for multiple comparison. Statistical significance was set at $P < 0.05$. R version 3.3.1 was used for statistical analyses.

3 RESULTS

3.1 Molecular typing of bacterial isolates and patient characteristics

MLST revealed that 36 of 50 (72%) *E. coli* O25 isolates were ST131. The antimicrobial susceptibilities of ST131 and non-ST131 are shown in Table 1. The ST131 group showed a significantly higher prevalence of antimicrobial non-susceptible isolates than the non-ST131 group. Based on *fimH*-based clonal typing, 36 ST131 isolates were classified into two genotypes: *fimH*30 (n = 31, 86%) and non-*fimH*30 (n = 5, 14%). Of the 5 non-*fimH*30 ST131 isolates, 3 were *fimH*22, whereas the others were 1 each of the *fimH*375 and *fimH*54 genotypes. There was one *fimH*30 isolate with ST3475, which differs from ST131 by a single nucleotide in *adk.* The 32 *fimH*30 isolates were further classified in 28 *H*30R1 isolates (87%), 3 *H*30Rx isolates (10%) and 1 *H*30S (3%) isolate. The *fimH*30 isolate with ST3475 belonged to *H*30R1. Among the 27 *H*30R1-ST131 isolates, 2 isolates, MRY09-619 and MRY09-620, possessed an 11,894-base pair (bp) region named as M27PP1 which was specific to the recently reported CTX-M-27-producing emerging clone C1-M27 (15).

3.2 Plasmid replicon types

Replicon types for 92 of the total 101 plasmids in this study, were detected by PlasmidFinder. The nine plasmids in which replicon types were not detected were referred to as non-detected replicon-type (ND) plasmids. The nine plasmid sequences of the ND plasmids were compared with completed sequences in the NCBI database using the *BLA*STN program. Results indicated

that 3 plasmids were similar to the replicon type IncN plasmid, pRSB201 (accession number JN1023417) with 72–78% coverage, 2 plasmids were also similar to replicon type IncN plasmid pN-Cit (accession number JQ996149) with 93% and 97% coverage, 1 plasmid was similar to replicon type IncX plasmid pYU39_IncX (accession number CP011431) with 71% coverage, and 1 plasmid was similar to replicon type IncP plasmid pHKSHmcr1_P2_p1 (accession number MF136778) with 83% coverage. The identity was 100% for all plasmids. Therefore, plasmids that had sequences with high similarity to the IncN, IncX, and IncP plasmids were referred to as IncN-like, IncX-like, and IncP-like plasmids, respectively. One of the other ND plasmids shared 99% identity and 86% query coverage with the phage-like plasmid, pECHO89 (accession number HG530657), isolated from *E. coli* H89.

All replicon types of plasmid DNA bands from 50 isolates, including the replicon sequence type (RST) for the IncF group, are shown in Fig. 1. The most prevalent replicon type was the multi-replicon IncF group of IncFII-FIA-FIB with the FAB formula F1:A2:B20. Additionally, among the 27 *H*30R1-ST131 isolates, 24 (89%) harbored F1:A2:B20 plasmid but among the 9 non-*H*30R1-ST131 isolates and 14 non-ST131 isolates, only 1 ST69 isolate did. The two C1-M27 clade isolates, MRY09-620 and MRY09-619, harbored the F1:A2:B20 plasmid (pMRY09- 620ECO_1) and F-:A2:B- plasmid (pMRY09-619ECO_1), respectively. Plasmids with a replicon type other than the IncF group and Col type were harbored by 18 isolates, of which IncZ (n = 6) was the most common followed by IncI1 (n = 4). The gene profile of plasmids harbored by 27 *H*30R1-ST131 isolates are shown in Fig. S2.

3.3 Antimicrobial resistance genes

3.3.1 Prevalence and location of antimicrobial resistance genes

Among the 36 ST131 isolates, 33 (92%) possessed at least one antimicrobial resistance gene, whereas only 5 (36%) of the 14 non-ST131 isolates did. This corresponded to the higher prevalence of antimicrobial non-susceptible isolates in the ST131 group compared with that in

the non-ST131 group. Furthermore, the higher prevalence of resistance genes among the ST131 groups was related to the high prevalence of resistance genes in F1:A2:B20 plasmids of *H*30R1-ST131. Comparison between F1:A2:B20 and the other plasmid types among the 36 ST131 isolates revealed significant differences in the prevalence of resistance genes. The most significant difference between the F1:A2:B:20 plasmids and others was the prevalence of *bla*TEM-1 (20/24, 83%), followed by aminoglycoside resistance genes [*aac(3)-IId* and *aadA5*], sulfamethoxazole-trimethoprim resistance genes [*sul1*, *sul2*, and *dfrA17*] and a macrolide resistance gene [*mph(A)*]; (Fig. 2, Fig. S2).

Among 5 non-ST131 isolates with antimicrobial resistance genes, 4 belonged to ST69 and the 1 was ST3475, which has a single nucleotide difference from ST131. All 5 isolates harbored *bla*TEM-1, which accounted for the only resistance gene in one of the ST69 isolates. Another ST69 isolate harbored *sul2* and *strA* with *bla*TEM-1 while the remaining two ST69 isolates co-harbor *tet(B)*, which was not found in ST131 isolates. Lastly, the ST3475 isolate co-harbored *aph(3')-Ia*, which was also not found in ST131 isolates, and *mph(A)*.

Six isolates with $bla_{\text{CTX-M}}$ were detected. Four *H*30R1-ST131 isolates harbored $bla_{\text{CTX-M-14}}$ on their plasmids and the replicon types and FAB formulae for the IncF group of these were F1:A2:B20 (n = 1), F4:A-:B- (n = 1), and IncZ (n = 2); (Fig. 1).

Two *H*30Rx-ST131 isolates harbored $bla_{\text{CTX-M-15}}$. One isolate harbored $bla_{\text{CTX-M-15}}$ on its IncFIA-R plasmid, which may be generated by fusion of F-:A1:B- with the IncR plasmid, and the other isolate harbored $bla_{\text{CTX-M-15}}$ on the chromosome.

3.3.2 Multidrug resistance region

The multidrug resistance region (MRR), consisting of approximately 35-kb, was found on a completed sequence of pMRY09-581ECO_1 (F1:A2:B20) and is shown in Fig. 3. The MRR consists of 3 regions: region I with *aadA5*, *sul1,* and *mph*; region II with *sul2*, *strA*, *strB*, and *tet(A)*; and region III with *dfrA17* and *intI1*. Region I was found in other 9 plasmids, all of which

were F1:A2:B20 (Fig.1). Among these 9 plasmids, 6 had inverted region III upstream of the *aadA5* of region I. This resulted in a typical class I integron with gene cassettes (*dfrA17, aadA5*) and 3′-conserved segment with genes *qacEΔ1* and *sul1*, which is In54 according to the INTEGRALL, integron database (http://integrall.bio.ua.pt/). Region II was found on four of 9 plasmids with region I, but all four had sequence gaps between region I. This was because they were assembled as different contigs by short read sequencing, which may have been because of the abundantly contained IS*26* within this MRR. In contrast, MRR may not be associated with $bla_{\text{CTX-M-14}}$, as although all four isolates with $bla_{\text{CTX-M-14}}$ had F1:A2:B20, only one of the four had the MRR. In addition, this isolate (MRY09-600) had $bla_{\text{CTX-M-14}}$ in its IncZ plasmid and not in the F1:A2:B20 plasmid. MRY09-576 had $bla_{\text{CTX-M-14}}$ in its F1:A2:B:20 plasmid (pMRY09-576ECO_1) but this plasmid did not have the MRR.

3.4 Characteristics of F1:A2:B20 plasmids

3.4.1 Size and similarity

The size of 24 F1:A2:B20 plasmids ranged from 86,535 to 159,781 bp with a median size of 124,952.5 bp. The smaller plasmids tended to lose some of the transfer genes, whereas larger plasmids reserved almost all transfer genes and also possessed antimicrobial resistance genes (Fig. S2a). Three F1:A2:B20 plasmids, pMRY09-581ECO_1, pMRY09-592ECO_1, and pMRY09-597ECO_1, were completely sequenced and the largest, pMRY09-581ECO_1, was used as a reference for comparative analysis of 24 F1:A2:B20 plasmids. All but one of the compared F1:A2:B20 plasmids shared high similarity sequences (100% identity, ≥93% coverage). One plasmid (pMRY09-588ECO_1) was less similar to the reference plasmid pMRY09-581ECO_1 because it had an approximately 32-kb inserted sequence associated with the IncN plasmid. The backbone sequence of pMRY09-588ECO_1, except for the inserted sequence, was highly similar to that of pMRY09-581ECO_1.

Linear maps of seven completely sequenced IncF group plasmids harbored by *H*30R1-ST131 are shown in Fig. 4. Three F1:A2:B20 plasmids were from this study, whereas the other three F1:A2:B20 plasmids and one F2:A2:B20 plasmid (F2 allele is different from F1 allele by 27 bp) were from the NCBI database. These seven plasmids were similar in overall structure even though they were from geographically different regions, wherein the three from this study were from Japan, two were from Seattle and Michigan (United States); (pG150_1 and pCA08, respectively), and one each were from Germany (pH105) and Thailand (pEC732_2). All plasmids, but one (pG150_1), from the public database had MRR with all three regions exhibiting the typical integron structure, whereas pG150_1 had only region I.

3.4.2 Plasmid stability genes and essential transfer genes

The presence of plasmid stability genes, encoding plasmid partitioning systems and toxin-antitoxin (TA)-based plasmid addiction systems, and transfer genes was compared between 24 F1:A2:B20 plasmids and 31 non-F1:A2:B20 plasmids harbored by 27 *H*30R1-ST131 isolates (Table 2; Fig. S2). Among the 31 non-F1:A2:B20 plasmids, 15 had IncF replicons (hereinafter, referred to as IncF-non-F1:A2:B20 plasmids). Although all F1:A2:B20 plasmids exhibited a plasmid-partitioning system, fewer IncF-non-F1:A2:B20 plasmids did so (87%) and only 4 (27%) of 16 non-IncF-type plasmids did so. Similar results were observed for TA-based plasmid addiction systems encoding genes, as all F1:A2:B20 plasmids had multiple systems, but much fewer were observed in IncF-non-F1:A2:B20 plasmids and none in non-IncFtype plasmids.

Based on reports related to their functions and mutational analyses of the conjugal transfer region, essential transfer genes are defined as genes absolutely required for conjugation of each plasmid replicon type (Fig. S2). Ten of 24 F1:A2:B20 plasmids possessed all essential transfer genes, whereas 28 of 31 non-F1:A2:B20 plasmids also possessed all essential transfer

genes (42% vs. 90%, respectively; $P < 0.05$). The transfer gene lost by most F1:A2:B20 plasmids was *traF*F, followed by *traQ*F which was associated with mating pair formation.

3.4.3 Stability and conjugation ability of F1:A2:B20 plasmids

Plasmid stability was assessed for one F1:A2:B20 plasmid, pMRY09-586ECO_1, and four non-F1:A2:B20 plasmids after being transferred into *E. coli* DH10B (Fig. 5). In the absence of ciprofloxacin, F1:A2:B20 and F35:A-:B- were stably maintained, whereas 0.3–1.3% of the population lost the F4:A-:B-, IncZ, and IncN plasmids. In the presence of ciprofloxacin, the F1:A2:B20 and IncN plasmids were still stably maintained for 20 days, whereas 0.7–33.7% of the population lost IncFII and IncZ during the 20 days. F1:A2:B20 was the only plasmid stably maintained for 20 days under both conditions.

The conjugation ability of seven F1:A2:B20 plasmids and five non-F1:A2:B20 plasmids possessing all essential transfer genes was assessed using in triplicate conjugal transfer experiments (Table 3). Five non-F1:A2:B20 consisted of three IncF (F35:A2:B20, F4:A-:B-, F35:A-B-) and one each of IncN and IncZ plasmids. All 5 non-F1:A2:B20 plasmids were self-transmissible and the averages of conjugation efficiency ranged from 1.67×10^{-2} to 3.48×10^{-4}. In contrast, no transconjugants were obtained from three of the seven isolates containing the F1:A2:B20 plasmid, although they carried all essential transfer genes. In addition, the conjugation efficiencies of the other four F1:A2:B20 plasmids ranged from 6.00×10^{-6} to 3.33×10^{-7}, which were lower than those of non-F1:A2:B20 plasmids. Any plasmids lacking at least one essential transfer gene were not transferred.

4 DISCUSSION

MLST of the 50 *E. coli* O25 isolates indicated that 36 (72%) were ST131. As approximately 15% of all *E. coli* isolates obtained from fecal samples of patients with diarrhea in Japan were O25 in

2008, approximately 10% of this population may have already carried *E. coli* ST131 in their digestive tract. Although the isolates utilized in this study were not subjected to antimicrobial selection, the ST131 group was found to be more multidrug-resistant than the non-ST131 group.

Further molecular typing revealed that most ST131 (78%; 28/36) were *H*30R1 and harbored multiple resistance genes, although only four of these carried $bla_{\text{CTX-M-14}}$. In contrast, of the three *H*30Rx isolates, two possessed $bla_{\text{CTX-M-15}}$. These findings were consistent with previous reports from other countries indicating that the *H*30R1-ST131 subclone was predominant among the ST131 population that did not carry $bla_{\text{CTX-M}}$ or did produce CTX-M-14/-27 in Japan, Korea, USA, Germany, and Spain (8, 12, 14, 28, 29), whereas the *H*30Rx subclone was dominant among the CTX-M-15-producing *E. coli* ST131 population (8, 15, 30, 31).

Recent analyses of 4,071 globally sourced genomes of the ST131 collection suggests that the clades, C1 and C2, which correspond to *H*30R1 and *H*30Rx, respectively, may have co-circulated globally for some time (32). However, $bla_{\text{CTX-M}}$ genes exhibit stronger chronological and geographical patterns. This indicates that the *H*30R1 and *H*30Rx clones circulated before acquiring $bla_{\text{CTX-M}}$, where some clones that acquired and stably maintained $bla_{\text{CTX-M}}$ dominant in each locality kept circulating with the gene. Examples of such representative clones may be *H*30Rx with $bla_{\text{CTX-M-15}}$ and *H*30R1 with $bla_{\text{CTX-M-27}}$.

The strength of this study is that the genomes of plasmids and chromosomes were separately sequenced and analyzed to clarify the location of antimicrobial resistance genes and replicon types of each plasmid. F1:A2:B20 plasmids were highly prevalent among *H*30R1-ST131, which harbored multiple resistance genes compared with other plasmid types. Notably, these resistance genes tended to accumulate in F1:A2:B20 plasmids, despite the *H*30R1-ST131

isolates harboring other plasmids. In non-*H*30R1-ST131 isolates, antimicrobial resistance genes were located in plasmids with various replicon types.

Considering the strong association between *H*30R1, F1:A2:B20 and multiple antimicrobial resistance genes, the characteristics of these plasmids were further investigated. Although plasmid sizes exhibited a wide range, nearly all F1:A2:B20 plasmids shared highly identical sequences. Comparison with F1:A2:B20 plasmids in the public database also showed a high level of similarities although no epidemiological link was found. It is unlikely that these plasmids were distributed via conjugation because they tended to lose their transfer genes. In contrast, F1:A2:B20 plasmids may be well-maintained in host cells because of the higher prevalence of genes encoding plasmid partitioning systems and multiple TA-based plasmid addiction systems. Maherault *et al*. reported the F2:A1:B- plasmid with $bla_{\text{CTX-M-15}}$ had better adaptation to *E. coli* which resulted in evolutionary success of global CTX-M-15-producing *E. coli* ST131 dissemination (33). Likewise, this stably maintained multidrug-resistant F1:A2:B20 plasmids may confer an additional advantage to *E. coli*, enabling it to become multidrug-resistant and circulate freely among the human population even without antimicrobial pressure and contribute $bla_{\text{CTX-M}}$ dissemination.

The isolates investigated in this study were collected in 2008, which was before the increase of CTX-M-27 in Japan. It is now reported that *E. coli H*30R1 harboring $bla_{\text{CTX-M-27}}$ on F1:A2:B20 plasmid has been spreading in Japan (34). In addition, there were 27 completely sequenced *E. coli* plasmids with $bla_{\text{CTX-M-27}}$ in public database and seven were F1:A2:B20. On the other hands, $bla_{\text{CTX-M-14}}$ are carried on various replicon type plasmids including F4:A-:B- and IncZ in this study. In addition, completely sequenced F1:A2:B20 plasmid with $bla_{\text{CTX-M-14}}$ could not be detected even in the NCBI database and pMRY09-576ECO_1 is so far the only F1:A2:B20 plasmid which could be confirmed by the current study as carrying $bla_{\text{CTX-M-14}}$. It is likely that $bla_{\text{CTX-M-14}}$ was not able to be maintained in F1:A2:B20 plasmid while $bla_{\text{CTX-M-27}}$ was stably

maintained in F1:A2:B20 and kept circulating with *H*30R1 This may be one reason that the number of CTX-M-27-producing *E. coli* has been increasing since 2010, and has now became one of the most prevalent CTX-M type (34-39).

In conclusion, the ST131-*H*30R1 clone carrying the multidrug-resistant F1:A2:B20 plasmid may have been disseminated before it acquired bla_{CTX-M} and became a reservoir for this gene. Several sub-lineages were classified, not only by the chromosomal genome, but also via plasmid replicons and antimicrobial resistance gene profiles. Some of these lineages disseminated after acquiring bla_{CTX-M}, which is dominant at each locality, representing the different molecular epidemiology of various countries. Further studies are needed to elucidate the mechanisms underlying the global dissemination of *H*30Rx-CTX-M-15 and *H*30R1-CTX-M-27 as opposed to other lineages of ST131 and bla_{CTX-M} combinations.

Data availability. Completed plasmids have been deposited in the DNA DataBank of Japan (DDBJ; https://www.ddbj.nig.ac.jp/ddbj/index-e.html) under the following accession numbers: pMRY09-581ECO_1, AP018456; pMRY09-597ECO_1, AP018457; pMRY09-592ECO_1, AP018458.

SUPPLEMENTAL MATERIAL

FigureS1, Figure S2, Table S1

DECLARATIONS

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Isolate No.	ST	*fimH* allele	H sequence serotype	CIP	CTX	CTX-M Type (location)	IncF																						non-IncF								
							FII-FIA-FIB							FII-FIB							FIA-FIB		FII								FIA									
							F1:A2:B20	F2:A1:B10	F2:A1:B63	F4:A1:B1	F29:A2:B20	F35:A2:B20	F36:A1:B20	F24:A-:B1	F29:A-:B10	F36:A-:B1	F95:A-:B1	FY3:A-:B63	$F_{(NT)}$:A-:B$_{(NT)}$	F-:A1:B1	F-:A2:B20	F2:A-:B-	F4:A-:B-	F6:A-:B-	F34:A-:B-	F35:A-:B-	F95:A-:B-	$F_{(NT)}$:A-:B-	F-:A2:B-	IncR-F-: A1:B-	IncZ	IncB	IncI1	IncN	IncX1	IncX4	Col8282	ND
MRY09-574	131	*H*30R1	4	R	S																																
MRY09-577	131	*H*30R1	4	R	S																																
MRY09-578	131	*H*30R1	4	R	S																																
MRY09-581	131	*H*30R1	4	R	S																																
MRY09-583	131	*H*30R1	4	R	S																																
MRY09-584	131	*H*30R1	4	R	S																																
MRY09-586	131	*H*30R1	4	R	S																																
MRY09-587	131	*H*30R1	4	R	S																																
MRY09-588	131	*H*30R1	4	R	S																																
MRY09-589	131	*H*30R1	4	R	S																																
MRY09-592	131	*H*30R1	4	R	S																																
MRY09-594	131	*H*30R1	4	R	S																																
MRY09-595	131	*H*30R1	4	R	S																																
MRY09-596	131	*H*30R1	4	R	S																																
MRY09-598	131	*H*30R1	4	R	S																																
MRY09-599	131	*H*30R1	4	R	S																																
MRY09-607	131	*H*30R1	4	R	S																																
MRY09-612	131	*H*30R1	4	R	S																																
MRY09-615	131	*H*30R1	4	R	S																																
MRY09-616	131	*H*30R1	4	R	S																																
MRY09-619	131	*H*30R1	4	R	S																																
MRY09-620	131	*H*30R1	4	R	S																																
MRY09-623	131	*H*30R1	4	R	S																																
MRY09-576	131	*H*30R1	4	R	R	14 (F1:A2:B20)																															
MRY09-593	131	*H*30R1	4	R	R	14 (F4:A-:B-)																															
MRY09-597	131	*H*30R1	4	R	R	14 (IncZ)																															
MRY09-600	131	*H*30R1	4	R	R	14 (IncZ)																															#
MRY09-580	131	*H*30Rx	4	R	S																																
MRY09-602	131	*H*30Rx	4	R	R	15 (IncR-F-:A1:B-)																															
MRY09-608	131	*H*30Rx	4	R	R	15 (Chr)																															
MRY09-610	131	*H*30S	4	S	S																																
MRY09-582	131	*H*22	4	S	S																																
MRY09-590	131	*H*22	4	S	S																																
MRY09-613	131	*H*22	4	S	S																																
MRY09-604	131	*H*54	4	S	S																																
MRY09-603	131	*H*375	4	S	S																																
MRY09-606	3475	*H*30R1	4	R	S																																
MRY09-617	69	*H*27	4	S	S																																
MRY09-621	69	*H*27	4	S	S																																
MRY09-611	69	*H*27	4	S	S																																
MRY09-575	69	*H*27	4	S	S																																
MRY09-601	69	*H*27	18	S	S																																
MRY09-614	69	*H*27	18	S	S																																
MRY09-622	95	*H*27	4	S	S																																
MRY09-585	96	*H*25	42	S	I																																
MRY09-591	472	*H*457	9	S	S																																
MRY09-609	648	Not found	42	S	S																																
MRY09-579	1491	*H*198	16	S	S																																
MRY09-605	3355	*H*58-like	25	S	S																																
MRY09-618	7965	*H*5	30	S	S																																

FIG 1. Distribution of IncF replicon sequence types among 50 diarrheal *Escherichia coli* isolates and characteristics of the isolates. ST, sequence type; *H*30R1 and *H*30Rx were distinguished by SNPs of *ybbW*. *H*58-like, *fimH* allele type differed by 5 bp from *H*58. ND, not detected; CIP, ciprofloxacin; CTX, cefotaxime; $F_{(NT)}$:A-:$B_{(NT)}$, non-typeable replicon sequence type of IncFII and IncFIB; Chr, chromosome. Colored cells indicate the presence of replicons. Red and pink indicates that plasmids possess resistance genes. Dark (red or black) colored cells in the F1:A2:B20 and IncI1 columns indicate the presence of Col156 or ΔCol156 replicons and light (pink or gray) colored cells indicate a plasmid without a replicon. # indicates that MRY09-600 had 2 plasmids whose replicons were not detected.

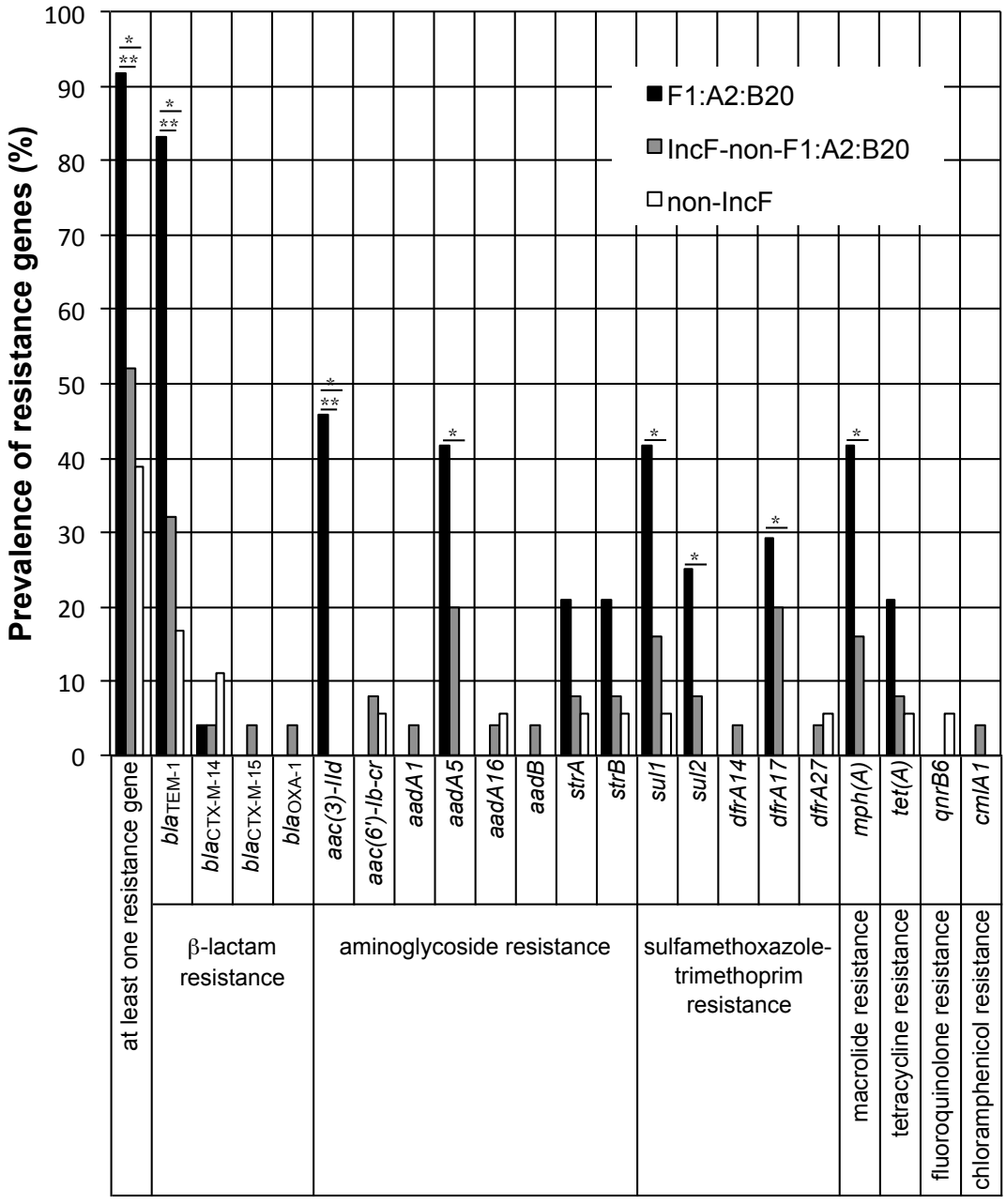


FIG 2. Comparison of the prevalence of antimicrobial resistance genes among 24 F1:A2:B20 plasmids, 25 IncF-non-F1:A2:B20 plasmids and 18 non-IncF plasmids harbored by ST131 isolates. * indicates a statistically significant difference between F1:A2:B20 and non-IncF plasmids. ** indicates a statistically

significant difference between F1:A2:B20 and IncF-non-F1:A2:B20 plasmids. Statistical analyses were performed using the Chi-square test with Tukey correction.

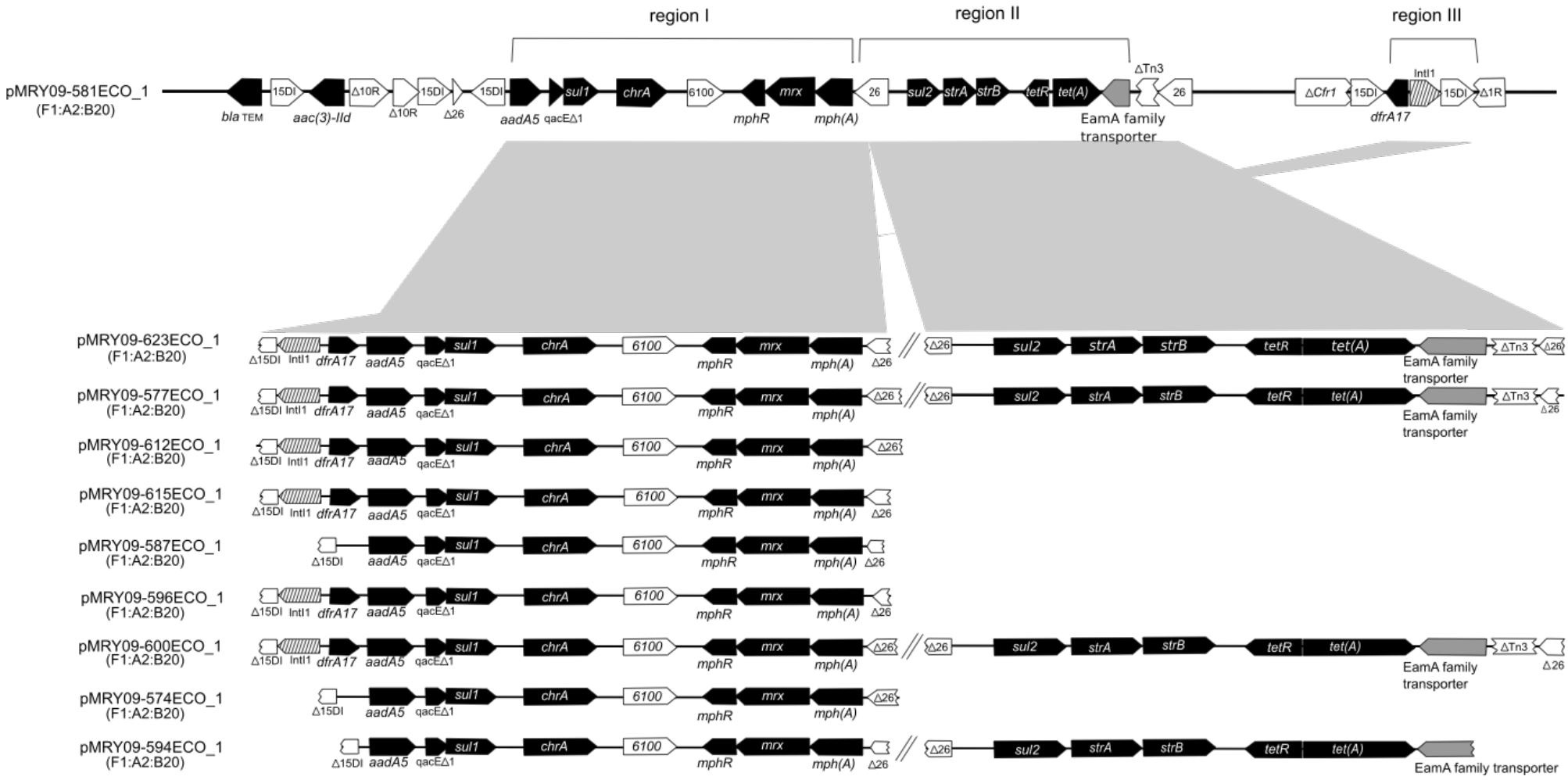


FIG 3. The multidrug resistance region (MRR) on pMRY09-581ECO_1 with common regions shared by 9 F1:A2:B20 plasmids. The uppermost

sequence is an approximately 35 kb MRR on the completely sequenced pMRY09-581ECO_1 (159,781 bp). The MRR consisted of 3 common regions of resistance genes shared by a maximum of 9 F1:A2:B20 plasmids. Region I consists of *aadA5-qacEΔ1-sul1-chrA*, which is a part of class 1 integron components, followed by IS*6100-mphR-mrx-mph(A)*. Region II consists of *sul2-strA-strB-tetR-tetA* flanked by 1 copy of IS*26*. Region III consists of *intI1* and *dfrA17*. Gray shades indicate shared backbone regions and resistance genes with a high degree of similarity (100% identical nucleotide sequences). Open reading frames are indicated by arrows: black, resistance genes; white with number, ISs and other mobile element genes; stripe pattern, IntI1 genes; gray, other genes. Double-slash marks (//) indicate sequence gaps within contigs of assembled plasmid sequences. IS*15DI* and IS*26* differ by 3 bp.

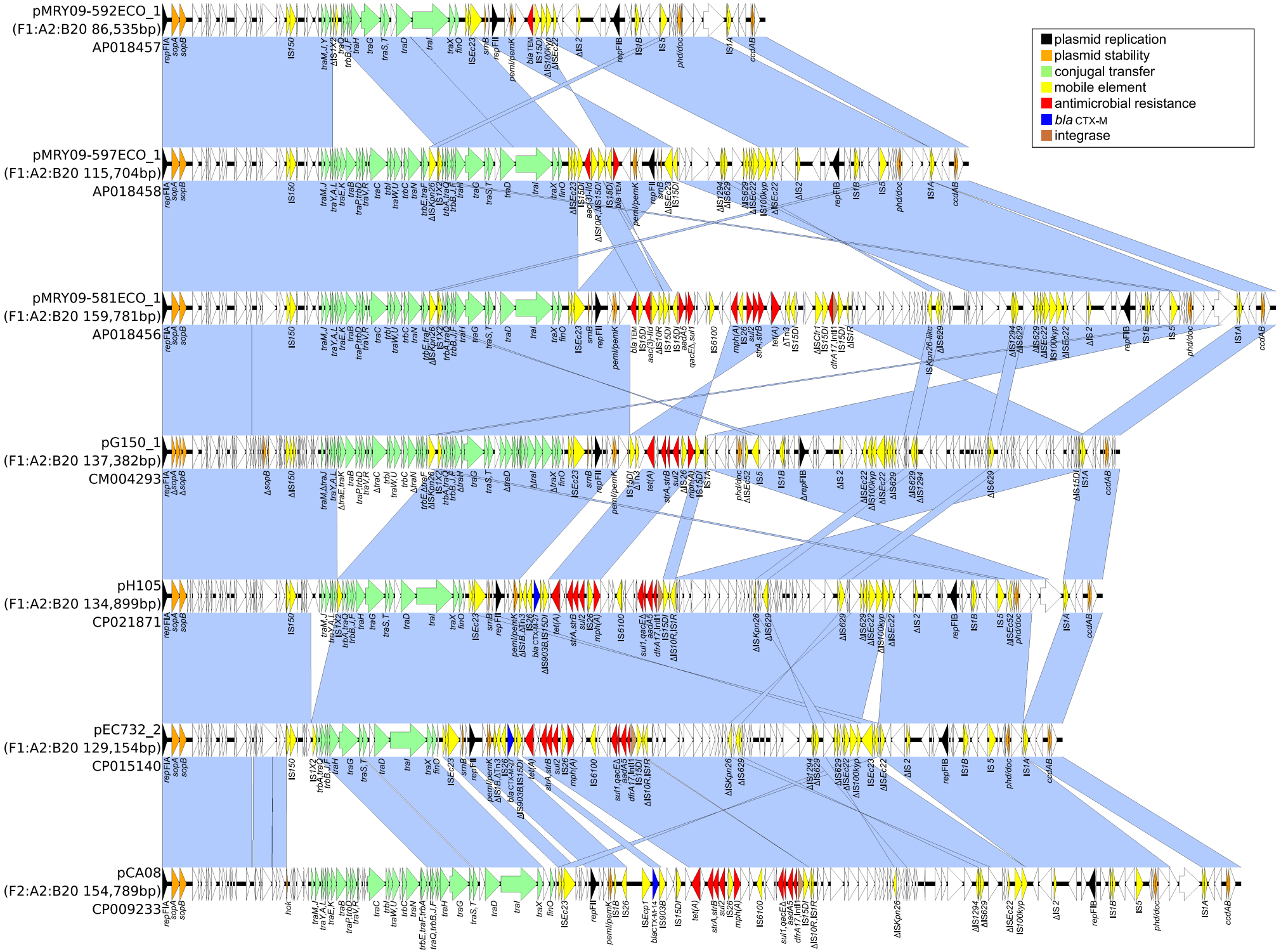
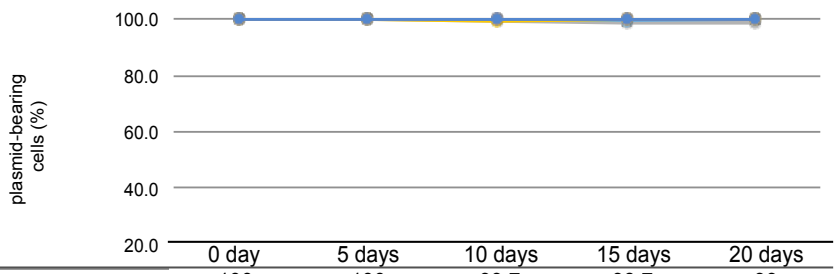

pMRY09-592ECO_1
(F1:A2:B20 86,535bp)
AP018457
pMRY09-597ECO_1
(F1:A2:B20 115,704bp)
AP018458
pMRY09-581ECO_1
(F1:A2:B20 159,781bp)
AP018456
pG150_1
(F1:A2:B20 137,382bp)
CM004293
pH105
(F1:A2:B20 134,899bp)
CP021871
pEC732_2
(F1:A2:B20 129,154bp)
CP015140
pCA08
(F2:A2:B20 154,789bp)
CP009233
plasmid replication
plasmid stability
conjugal transfer
mobile element
antimicrobial resistance
bla CTX-M
integrase

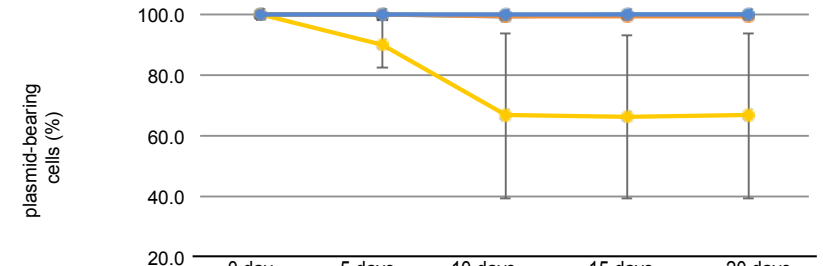
FIG 4. Linear maps of completed F1:A2:B20 and F2:A2:B20 plasmids from *fimH*30-ST131 *E. coli*. pMRY09-592ECO_1 (accession number AP018457), pMRY09-597ECO_1 (accession number AP018458) and pMRY09-581ECO_1 (accession number AP018456) were obtained in our study. The sequence data of pG150 (accession number CM004293), pH105 (accession number CP021871), pEC732_2 (accession number CP015140) and pCA08 (accession number CP009233) were obtained from the NCBI database. pG150 and pCA08 were isolated from ST131 *H*30R1 and pH105 and pEC732_2 were isolated from ST131 *H*30R1 with M27PP1 that was specific to recently reported CTX-M-27-producing emerging clone C1-M27. A subtype of the FII replicon, the F2 allele differs from the F1 allele by 27 bp. Open reading frames (ORFs) are portrayed by arrows. Genes involved with plasmid replication, plasmid stability, and plasmid conjugal transfers are indicated by black, orange, and light green arrows, respectively. Yellow and red arrows indicate mobile element genes and antimicrobial resistance genes. Blue and brown arrows indicate bla_{CTX-M} and integrase genes. Δ indicates disrupted genes. IS*15DI* differs by 3 bp from IS*26*.

a) without ciprofloxacin



	0 day	5 days	10 days	15 days	20 days
E.coli DH10B (IncN, pMRY09-594ECO_3)	100	100	99.7	98.7	99
E.coli DH10B (IncZ, pMRY09-597ECO_2)	100	100	100	99.7	100
E.coli DH10B (F4:A-:B-, pMRY09-593ECO_2)	100	100	99.3	100	100
E.coli DH10B (F35:A-:B-, pMRY09-586ECO_2)	100	100	100	100	100
E.coli DH10B (F1:A2:B20, pMRY09-586ECO_1)	100	100	100	100	100

b) with ciprofloxacin



	0 day	5 days	10 days	15 days	20 days
E.coli DH10B (IncN, pMRY09-594ECO_3)	100	100	100	100	100
E.coli DH10B (IncZ, pMRY09-597ECO_2)	100	100	99.3	100	100
E.coli DH10B (F4:A-:B-, pMRY09-593ECO_2)	100	90.3	66.7	66.3	66.7
E.coli DH10B (F35:A-:B-, pMRY09-586ECO_2)	100	100	99.7	99.3	99.3
E.coli DH10B (F1:A2:B20, pMRY09-586ECO_1)	100	100	100	100	100

FIG 5. Comparison of stability between F1:A2:B20 and other types of plasmids. Conjugants DH10B *E. coli* harboring each plasmid were used for plasmid stability experiments. Triplicated plasmid stability experiments were performed with or without exposure to 0.001 μg/ml of ciprofloxacin (CIP). Plasmid stability was evaluated by calculating the percentage of plasmid-bearing cells. In the absence of CIP, F1:A2:B20 and F35:A-:B- plasmids were maintained more stably for 20 days, whereas in the presence of CIP, F1:A2:B20 and IncN

plasmids were maintained more stably for 20 days. F1:A2:B20 plasmids were the most stably maintained regardless of CIP presence.

TABLE 1 Comparison of antimicrobial non-susceptibility between ST131 and non-ST131 isolates.

	number of isolates (%)		
Antimicrobial	ST131 (n = 36)	non-ST131 (n = 14)	*P* value
ampicillin	30 (83.3)	5 (35.7)	< 0.05
ampicillin/sulbactam	30 (83.3)	5 (35.7)	< 0.05
piperacillin/tazobactam	1 (2.8)	0	1
cefotaxime	6 (16.7)	1 (7.1)	0.657
ceftazidime	2 (5.6)	0	1
cefepime	6 (16.7)	0	0.167
ciprofloxacin	30 (83.3)	1 (7.1)	< 0.05
amikacin	0	0	0.28
gentamycin	13 (36.1)	0	< 0.05
tobramycin	14 (38.9)	0	< 0.05
minocycline	4 (11.1)	3 (21.4)	0.384
sulfamethoxazole/trimethoprim	15 (41.7)	1 (7.1)	< 0.05

TABLE 2 Prevalence of partitioning system genes, toxin-antitoxin (TA)-based plasmid addiction system genes and essential transfer genes among F1:A2:B20 plasmids and other replicon-types of plasmids harbored by 27 *H*30R1 isolates.

	Number of isolates (%)			*P* value
Genes on plasmids	F1:A2:B20 (n = 24)	non-F1:A2:B20 IncF (n = 15)	non-IncF types (n = 16)	Overall
partitioning systems	24 (100%)	13 (87%)	4 (25%)	< 0.05
TA-based plasmid addiction systems				
≥ 2 systems	24 (100%)	2 (13%)	0	
1 system	0	9 (60%)	6 (38%)	
0 system	0	4 (27%)	10 (63%)	< 0.05
essential transfer genes	10 (42%)	12 (80%)	16 (100%)	< 0.05

TABLE 3 Self-transmissible ability of F1:A2:B20 plasmids comparing to other replicon types of plasmids.

	Plasmid No.	Replicon type	FAB formula	Size (bp)	CTX-M type	Average of conjugation efficiency
all essential *tra* genes +	pMRY09-581ECO_1	FII-FIA-FIB-DCol156	F1:A2:B20	159,781		6.00×10^{-6}
	pMRY09-584 ECO_1			131,838		1.85×10^{-6}
	pMRY09-587 ECO_1			145,903		0
	pMRY09-598 ECO_1			136,880		3.33×10^{-7}
	pMRY09-607 ECO_1			119,934		0
	pMRY09-612 ECO_1			151,377		8.12×10^{-7}
	pMRY09-615 ECO_1			146,213		0
	pMRY09-616 ECO_1	FII-FIA-FIB-Col156	F35:A2:B20	128,573		4.57×10^{-4}
	pMRY09-593 ECO_2	FII	F4:A-:B-	71,351	14	4.18×10^{-4}
	pMRY09-586 ECO_2	FII	F35:A-:B-	73,703		2.79×10^{-3}
	pMRY09-594 ECO_3	N		55,848		1.67×10^{-2}
	pMRY09-597 ECO_2	Z		94,192	14	3.48×10^{-4}
all essential *tra* genes –	pMRY09-576 ECO_1	FII-FIA-FIB-DCol156	F1:A2:B20	124,412	14	0
	pMRY09-623 ECO_1	FII-FIA-FIB-DCol156	F1:A2:B20	157,841		0
	pMRY09-596 ECO_1	FII-FIA-FIB-DCol156	F1:A2:B20	125,842		0
	pMRY09-619 ECO_1	FIA-Col156	F-:A2:B-	88,810		0

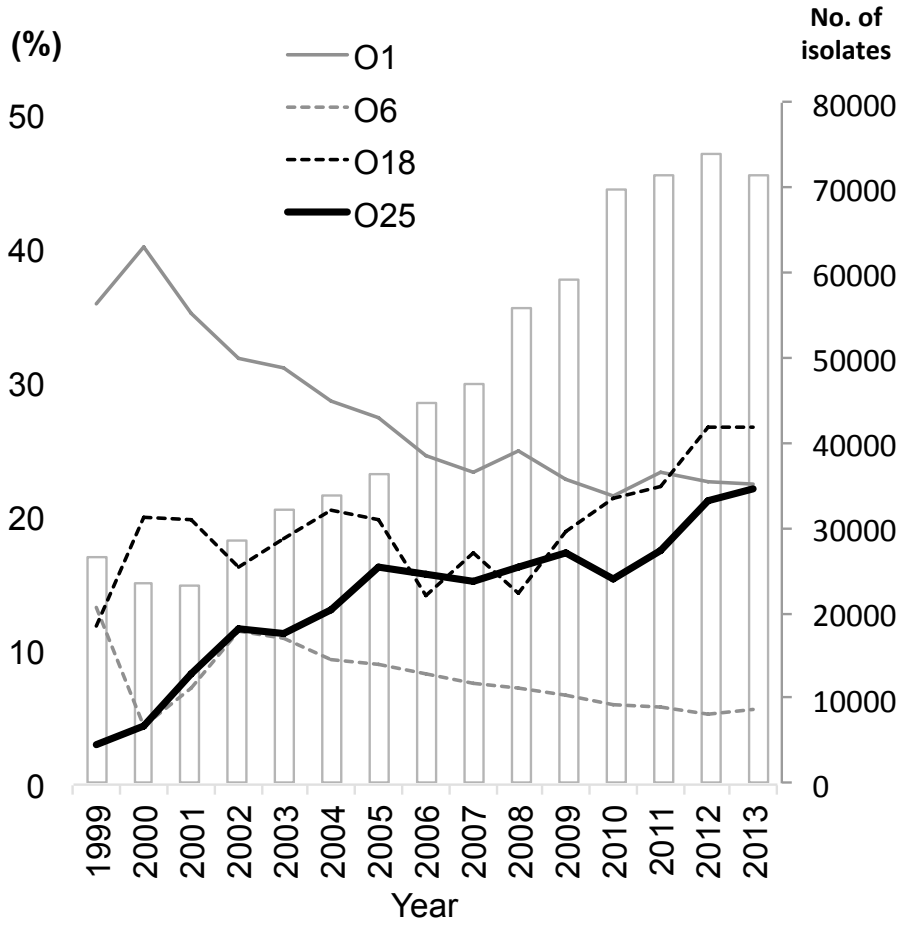
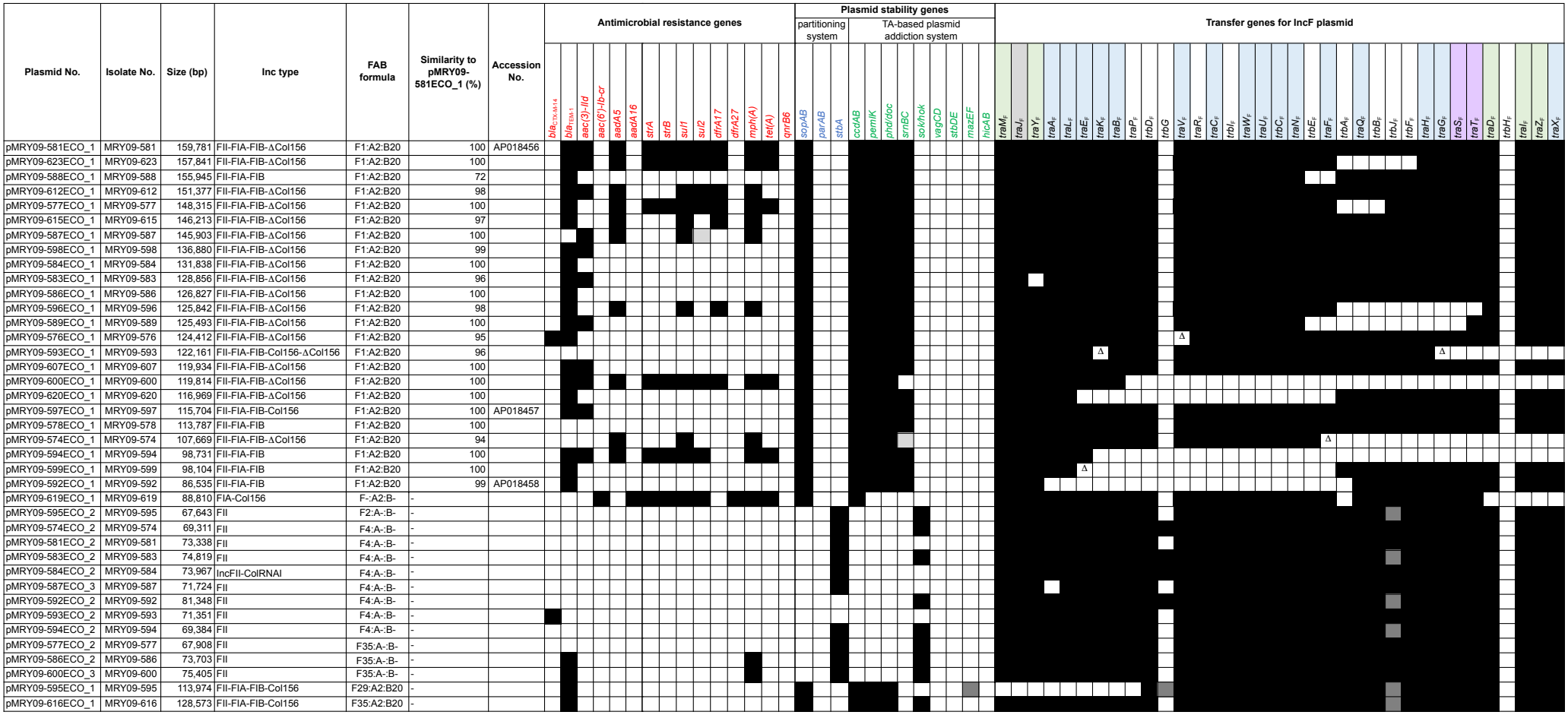


FIG S1. Prevalence of major O serotypes (O1, O6, O18 and O25) of diarrheagenic *E. coli* isolates from 1999 to 2013 in Japan. This graph was obtained from the Bio Medical Laboratory database (http://www.bml.co.jp/bct_info/c/index.html).

a) IncF plasmids harbored by *H*30R1 isolates.



Plasmid No.	Isolate No.	Size (bp)	Inc type	FAB formula	Similarity to pMRY09-581ECO_1 (%)	Accession No.
pMRY09-581ECO_1	MRY09-581	159,781	FII-FIA-FIB-ΔCol156	F1:A2:B20	100	AP018456
pMRY09-623ECO_1	MRY09-623	157,841	FII-FIA-FIB-ΔCol156	F1:A2:B20	100	
pMRY09-588ECO_1	MRY09-588	155,945	FII-FIA-FIB	F1:A2:B20	72	
pMRY09-612ECO_1	MRY09-612	151,377	FII-FIA-FIB-ΔCol156	F1:A2:B20	98	
pMRY09-577ECO_1	MRY09-577	148,315	FII-FIA-FIB-ΔCol156	F1:A2:B20	100	
pMRY09-615ECO_1	MRY09-615	146,213	FII-FIA-FIB-ΔCol156	F1:A2:B20	97	
pMRY09-587ECO_1	MRY09-587	145,903	FII-FIA-FIB-ΔCol156	F1:A2:B20	100	
pMRY09-598ECO_1	MRY09-598	136,880	FII-FIA-FIB-ΔCol156	F1:A2:B20	99	
pMRY09-584ECO_1	MRY09-584	131,838	FII-FIA-FIB-ΔCol156	F1:A2:B20	100	
pMRY09-583ECO_1	MRY09-583	128,856	FII-FIA-FIB-ΔCol156	F1:A2:B20	96	
pMRY09-586ECO_1	MRY09-586	126,827	FII-FIA-FIB-ΔCol156	F1:A2:B20	100	
pMRY09-596ECO_1	MRY09-596	125,842	FII-FIA-FIB-ΔCol156	F1:A2:B20	98	
pMRY09-589ECO_1	MRY09-589	125,493	FII-FIA-FIB-ΔCol156	F1:A2:B20	100	
pMRY09-576ECO_1	MRY09-576	124,412	FII-FIA-FIB-ΔCol156	F1:A2:B20	95	
pMRY09-593ECO_1	MRY09-593	122,161	FII-FIA-FIB-Col156-ΔCol156	F1:A2:B20	96	
pMRY09-607ECO_1	MRY09-607	119,934	FII-FIA-FIB-ΔCol156	F1:A2:B20	100	
pMRY09-600ECO_1	MRY09-600	119,814	FII-FIA-FIB-ΔCol156	F1:A2:B20	100	
pMRY09-620ECO_1	MRY09-620	116,969	FII-FIA-FIB-ΔCol156	F1:A2:B20	100	
pMRY09-597ECO_1	MRY09-597	115,704	FII-FIA-FIB-Col156	F1:A2:B20	100	AP018457
pMRY09-578ECO_1	MRY09-578	113,787	FII-FIA-FIB	F1:A2:B20	100	
pMRY09-574ECO_1	MRY09-574	107,669	FII-FIA-FIB-ΔCol156	F1:A2:B20	94	
pMRY09-594ECO_1	MRY09-594	98,731	FII-FIA-FIB	F1:A2:B20	100	
pMRY09-599ECO_1	MRY09-599	98,104	FII-FIA-FIB	F1:A2:B20	100	
pMRY09-592ECO_1	MRY09-592	86,535	FII-FIA-FIB	F1:A2:B20	99	AP018458
pMRY09-619ECO_1	MRY09-619	88,810	FIA-Col156	F-:A2:B-	-	
pMRY09-595ECO_2	MRY09-595	67,643	FII	F2:A-:B-	-	
pMRY09-574ECO_2	MRY09-574	69,311	FII	F4:A-:B-	-	
pMRY09-581ECO_2	MRY09-581	73,338	FII	F4:A-:B-	-	
pMRY09-583ECO_2	MRY09-583	74,819	FII	F4:A-:B-	-	
pMRY09-584ECO_2	MRY09-584	73,967	IncFII-ColRNAI	F4:A-:B-	-	
pMRY09-587ECO_3	MRY09-587	71,724	FII	F4:A-:B-	-	
pMRY09-592ECO_2	MRY09-592	81,348	FII	F4:A-:B-	-	
pMRY09-593ECO_2	MRY09-593	71,351	FII	F4:A-:B-	-	
pMRY09-594ECO_2	MRY09-594	69,384	FII	F4:A-:B-	-	
pMRY09-577ECO_2	MRY09-577	67,908	FII	F35:A-:B-	-	
pMRY09-586ECO_2	MRY09-586	73,703	FII	F35:A-:B-	-	
pMRY09-600ECO_3	MRY09-600	75,405	FII	F35:A-:B-	-	
pMRY09-595ECO_1	MRY09-595	113,974	FII-FIA-FIB-Col156	F29:A2:B20	-	
pMRY09-616ECO_1	MRY09-616	128,573	FII-FIA-FIB-Col156	F35:A2:B20	-	

b) IncZ and IncI plasmids harbored by *H*30R1 isolates.

Plasmid No.	**Isolate No.**	**Size (bp)**	**Inc type**	**Antimicrobial resistance genes**															**Plasmid stability genes**												**Transfer genes for IncI plasmid**																												
																			partitioning system			TA-based plasmid addiction system																																					
				$bla_{CTX-M-14}$	bla_{TEM-1}	*aac(3)-IId*	*aac(6')-Ib-cr*	*aadA5*	*aadA16*	*strA*	*strB*	*sul1*	*sul2*	*dfrA17*	*dfrA27*	*mph(A)*	*tet(A)*	*qnrB6*	*sopAB*	*parAB*	*stbA*	*ccdAB*	*pemIK*	*phd/doc*	*srnBC*	*sok/hok*	*vagCD*	*stbDE*	*mazEF*	*hicAB*	$traA_I$	$traB_I$	$traC_I$	$traD_I$	$traE_I$	$traF_I$	$traI_I$	$traJ_I$	$traK_I$	*sogL*	$traL_I$	$traM_I$	$traN_I$	$traO_I$	$traP_I$	$traQ_I$	$traR_I$	$traS_I$	$traT_I$	$traU_I$	$traV_I$	$traW_I$	$traX_I$	$traY_I$	$trbA_I$	$trbB_I$	$trbC_I$	*nikB*	*nikA*
pMRY09-597ECO_2	MRY09-597	94,192	Z	■																												■	■		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
pMRY09-600ECO_2	MRY09-600	101,196	Z	■																												■	■		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
pMRY09-587ECO_2	MRY09-587	94,744	I1-Col156																												■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
pMRY09-615ECO_2	MRY09-615	94,261	I1-Col156																												■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	▒
pMRY09-623ECO_2	MRY09-623	101,726	I1-Col156		■															■											■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
pMRY09-596ECO_2	MRY09-596	93,061	I1		■															■											■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■

c) IncN plasmids harbored by *H*30R1 isolates.

Plasmid No.	Isolate No.	Size (bp)	Inc type	Antimicrobial resistance genes															Plasmid stability genes									Transfer genes for IncN plasmid													
																			partitioning system			TA-based plasmid addiction system																			
				$bla_{CTX-M-14}$	bla_{TEM-1}	*aac(3)-IId*	*aac(6')-Ib-cr*	*aadA5*	*aadA16*	*strA*	*strB*	*sul1*	*sul2*	*dfrA17*	*dfrA27*	*mph(A)*	*tet(A)*	*qnrB6*	*sopAB*	*parAB*	*stbA*	*ccdAB*	*pemIK*	*phd/doc*	*smBC*	*sok/hok*	*vagCD*	*stbDE*	*mazEF*	*hicAB*	$traL_N$	$traM_N$	$traA_N$	$traB_N$	$traC_N$	$traS_N$	$traD_N$	$traE_N$	$traO_N$	$traF_N$	$traG_N$	$traI_N$	$traJ_N$	$traK_N$
pMRY09-594ECO_3	MRY09-594	55,848	N				■		■			■			■		■	■			■																				
pMRY09-586ECO_3	MRY09-586	44,042	ND (IncN-like)							■	■																			■	■	■	■	■	■	■	■	■	■	■	■	■	■
pMRY09-587ECO_4	MRY09-587	40,754	ND (IncN-like)																									▒		■	■	■	■	■	■	■	■	■	■	■	■	■	■
pMRY09-596ECO_3	MRY09-596	34,199	ND (IncN-like)																									■		■	■	■	■	■	■	■	■	■	■	■	■	■	■
pMRY09-597ECO_3	MRY09-597	42,702	ND (IncN-like)																									▒		■	■	■	■	■	■	■	■	■	■	■	■	■	■
pMRY09-600ECO_5	MRY09-600	34,869	ND (IncN-like)																		■							■		■	■	■	■	■	■	■	■	■	■	■	■	■	■

d) IncX plasmids harbored by *H*30R1 isolates.

Plasmid No.	Isolate No.	Size (bp)	Inc type	Antimicrobial resistance genes														Plasmid stability genes: partitioning system			Plasmid stability genes: TA-based plasmid addiction system							Transfer genes for IncX plasmid												
				$bla_{CTX-M-14}$	bla_{TEM-1}	*aac(3)-IId*	*aac(6')-Ib-cr*	*aadA5*	*aadA16*	*strA*	*strB*	*sul1*	*sul2*	*dfrA17*	*dfrA27*	*mph(A)*	*tet(A)*	*qnrB6*	*sopAB*	*parAB*	*stbA*	*ccdAB*	*pemIK*	*phd/doc*	*srnBC*	*sok/hok*	*vagCD*	*stbDE*	*mazEF*	*hicAB*	*virD2*	*virD4*	*virB1*	*virB2*	*VirB3*	*virB4*	*virB5*	*virB6*	*virB7*	*virB8*	*virB9*	*virB10*	*virB11*
pMRY09-592ECO_3	MRY09-592	34,433	X1																																					
pMRY09-589ECO_2	MRY09-589	33,503	X4																																					
pMRY09-598ECO_2	MRY09-598	33,765	X4																																					
pMRY09-600ECO_4	MRY09-600	36,337	ND (IncX-like)																																					

Amino acid (coverage cut off 80%)
- identity>95%
- identity>90%
- identity>80%
- identity>70%

ND, not detected

Function of transfer genes
- mating pair formation
- DNA transfer
- transcription regulator
- exclusion system
- DNA synthesis
- essential but unknown function
- unknown

FIG S2. Distribution of resistance genes, plasmid stability genes, and transfer genes harbored by plasmids in *H*30R1. Black and gray cells indicate the presence of each gene. The lighter the gray, the less identical the gene is to a reference gene. Δ indicates that the gene is truncated and disrupted by insertion of ISs. Colored columns of transfer genes for each plasmid replicon type indicate essential transfer genes defined as genes

required for conjugal transformation of each Inc-type plasmid according to previous reports regarding their functions and the adverse effects of mutations in transfer genes (supplemental reference 1-5). The sequence's similarity to the pMRY09-581ECO_1 plasmid was obtained using the inter plasmid analyzing tool (iPAT) established by the Pathogen Genomics Center at the National Institute of Infectious Diseases in Japan. The following sequences were evaluated to confirm the presence of genes with several annotation candidates and were missed by annotations using the Prodigal program (version 2.60): *sok*, CAA29258; *mazE*, AKM38048; *mazF*, AKM38051; *srnC*, JX077110; *traY*, AAR25080; *trbG*, BAI28839; *traV*, AMX43309; *traR*, AJO86965; *trbJ*, AJO86976; *nikA*, AMX17185; *virB7*, AEK49305 and AEQ61781. The mechanisms of TA-based plasmid stability addiction systems involve inhibition of DNA gyrase for *ccdAB*, ribosome-independent mRNA cleavage for *pemIK*, binding to the 30S ribosomal subunit for *phd/doc*, damage of the cell membrane for *srnBC* and *sok/hok*, cleavage of tRNA for *vagCD*, cleavage of ribosome-bound mRNA for *stbDE,* ribosome-independent mRNA cleavage and cleavage of 16S rRNA for *mazEF*, ribosome-independent mRNA cleavage for *hicAB* (supplemental reference 6-10) ND, not detected.

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Table S1 Prevalence of intact genes related to partitioning system and toxin-antitoxin (TA)-based plasmid addiction system and essential and intact genes related to conjugal transfer among F1:A2:B20 plasmids and other plasmids harbored by 27 *H*30R1 isolates.

	Number of isolates				
		non-F1:A2:B20 IncF	non-IncF types (n = 16)		
Genes on plasmids	F1:A2:B20 (n = 24)	(n = 15)	IncZ and IncI (n = 6)	IncN (n = 6)	IncX (n = 4)
Partitioning system					
sopAB	24	3	0	0	0
stbA	0	10	0	2	0
parAB	0	0	2	0	0
TA-based plasmid addiction systems					
ccdAB	24	3	0	0	0
pemIK	24	2	0	0	0
phd/doc	24	2	0	0	0
srnBC	23	0	0	0	0
sok/hok	0	9	0	0	0
vagCD	0	0	0	0	0
stbDE	0	0	0	0	1
mazEF	0	1	0	4	0
hicAB	0	0	0	0	1
Essential transfer genes for IncF plasmid					
$traM_F$	24	14	-	-	-
$traJ_F$	24	14	-	-	-
$traY_F$	23	14	-	-	-
$traA_F$	23	13	-	-	-
$traL_F$	23	14	-	-	-
$traE_F$	21	14	-	-	-
$traK_F$	19	14	-	-	-
$traB_F$	20	14	-	-	-
$traV_F$	18	15	-	-	-
$traC_F$	19	15	-	-	-
$traW_F$	19	15	-	-	-
$traU_F$	19	15	-	-	-
$trbC_F$	19	15	-	-	-
$traN_F$	19	15	-	-	-
$traF_F$	16	15	-	-	-
$traQ_F$	17	15	-	-	-
$traH_F$	19	15	-	-	-
$traG_F$	18	15	-	-	-
$traS_F$	18	15	-	-	-
$traT_F$	19	15	-	-	-
$traD_F$	20	14	-	-	-
$traI_F$	20	14	-	-	-
$traZ_F$	20	14	-	-	-
$traX_F$	20	14	-	-	-
Essential transfer genes for IncZ and IncI plasmid					
$traB_I$	-	-	6	-	-
$traC_I$	-	-	6	-	-
$traI_I$	-	-	6	-	-
$traJ_I$	-	-	6	-	-
$traK_I$	-	-	6	-	-
sogL	-	-	6	-	-
$traL_I$	-	-	6	-	-

traM$_I$	-	-	6	-	-
traN$_I$	-	-	6	-	-
traO$_I$	-	-	6	-	-
traP$_I$	-	-	6	-	-
traQ$_I$	-	-	6	-	-
traR$_I$	-	-	6	-	-
traT$_I$	-	-	6	-	-
traU$_I$	-	-	6	-	-
traV$_I$	-	-	6	-	-
traW$_I$	-	-	6	-	-
traX$_I$	-	-	6	-	-
traY$_I$	-	-	6	-	-
trbA$_I$	-	-	6	-	-
trbB$_I$	-	-	6	-	-
trbC$_I$	-	-	6	-	-
nikB	-	-	6	-	-
nikA	-	-	6	-	-
Essential transfer genes for IncN plasmid					
traL$_N$	-	-	-	6	-
traM$_N$	-	-	-	6	-
traA$_N$	-	-	-	6	-
traB$_N$	-	-	-	6	-
traC$_N$	-	-	-	6	-
traS$_N$	-	-	-	6	-
traD$_N$	-	-	-	6	-
traE$_N$	-	-	-	6	-
traO$_N$	-	-	-	6	-
traF$_N$	-	-	-	6	-
traG$_N$	-	-	-	6	-
traI$_N$	-	-	-	6	-
traJ$_N$	-	-	-	6	-
Essential transfer genes for IncX plasmid					
virD2	-	-	-	-	4
virD4	-	-	-	-	4
virB1	-	-	-	-	4
virB2	-	-	-	-	4
virB3	-	-	-	-	4
virB4	-	-	-	-	4
virB5	-	-	-	-	4
virB6	-	-	-	-	4
virB7	-	-	-	-	4
virB8	-	-	-	-	4
virB9	-	-	-	-	4
virB10	-	-	-	-	4
virB11	-	-	-	-	4