JAC Antimicrob Resist
https://doi.org/10.1093/jacamr/dlad106

JAC-Antimicrobial Resistance

Analysis of the stepwise acquisition of *bla*$_{CTX-M-2}$ and subsequent acquisition of either *bla*$_{IMP-1}$ or *bla*$_{IMP-6}$ in highly conserved IncN-pST5 plasmids

Kazuko Ikegaya[1,2], Kotaro Aoki[3], Kohji Komori [1], Yoshikazu Ishii[1,3]* and Kazuhiro Tateda[1,3]

[1]*Department of Microbiology and Infection Control and Prevention, Toho University Graduate School of Medicine, Tokyo, Japan;* [2]*Department of Clinical Laboratory, Shizuoka City Shimizu Hospital, Shizuoka, Japan;* [3]*Department of Microbiology and Infectious Diseases, Toho University School of Medicine, 5-21-16 Omori-nishi, Ota-ku, Tokyo 143-8540, Japan*

*Corresponding author. E-mail: yishii@med.toho-u.ac.jp

Received 31 August 2023; accepted 12 September 2023

Objectives: ESBL and carbapenemase genes in Enterobacterales spread via plasmids. Nosocomial outbreaks caused by Enterobacterales producing both CTX-M-2 and either IMP-1 or IMP-6-type carbapenemases have been reported. These organisms carry the incompatibility type N plasmid belonging to plasmid ST 5 (IncN-pST5). We investigated the construction process of the ESBL and carbapenemase genes co-carrying IncN-pST5.

Methods: We retrospectively performed draft WGS analysis for *bla*$_{IMP}$- or *bla*$_{CTX-M}$-positive Enterobacterales in our strain collection ($n=281$).

Results: We selected four types of *Escherichia coli* plasmids for our study: type A, which carries both *bla*$_{CTX-M-2}$ and *bla*$_{IMP-1}$ ($n=6$); type B, which carries both *bla*$_{CTX-M-2}$ and *bla*$_{IMP-6}$ ($n=2$); type C, which carries *bla*$_{CTX-M-2}$ ($n=10$); and type D, which carries no β-lactamase genes ($n=1$). It should be noted that type D plasmid was only detected in *E. coli* TUM2805, which carries the *bla*$_{CTX-M-14}$ on the IncB/O/B/Z plasmid. Long-read sequencing using MinION revealed that all types of IncN-pST5 were highly conserved and carried a class 1 integron. Integron numbers were type A for In798, type B for In1690, type C for In127 and type D for In207. Because the gene cassettes downstream of *bla*$_{IMP}$ were different between In798 and In1690, the change from *bla*$_{IMP-1}$ to *bla*$_{IMP-6}$ by point mutation was unlikely. Representative plasmids from types A, B and C were conjugatively transferred with quite a high frequency between 1.3×10^{-1} and 2.5×10^{-2}.

Conclusions: This study suggested that IncN-pST5 acquired *bla*$_{CTX-M-2}$ by IS*Ecp1* in a stepwise manner, followed by either *bla*$_{IMP-1}$ or *bla*$_{IMP-6}$ into a class 1 integron.

Introduction

When a strain simultaneously harbours an ESBL gene and a carbapenemase gene, these genes are expressed simultaneously, contributing to resistance to broad-spectrum cephalosporins and carbapenems in Enterobacterales.[1] This multi-β-lactam resistance can be attributed to horizontal gene transfer (HGT), where a single plasmid replicon carries both ESBL and carbapenemase genes. Mobile genetic elements (MGEs) such as ISs, integrons and transposons mediate the inter-replicon transfer of β-lactamase genes.[2]

One such IS, IS*Ecp1*, facilitates the transfer of the CTX-M-type ESBL gene, *bla*$_{CTX-M}$, from the *Kluyvera* spp. chromosome to various plasmids and provides a promoter to express it.[3,4] IS*Ecp1* can transpose to various replicons, not only plasmids but also chromosomes, and locate on them.[5] On the other hand, integrons accumulate characteristic antimicrobial resistance genes through site-specific recombination and provide a promoter to express their gene clusters. An integron contains 5′ and 3′-end conservative sequences called 5′CS and 3′CS. The 5′CS, consisting of *intI*, provides a promoter for the expression of gene cassettes located downstream.[6] IntI is an enzyme that catalyses site-specific recombination between *attI* and *attC* sites located next to the antimicrobial resistance gene. The 3′CS consists of sulphonamide resistance gene *sul1* and an incomplete quaternary ammonium salt resistance gene *qacE*Δ1.

Frequent co-localization is observed between carbapenemase genes and MGEs. Interestingly, class 1 integrons carry multiple types of carbapenemase genes, such as bla_{IMP}, bla_{VIM} and bla_{GES}, among others.[6–11] Carbapenemase gene-carrying plasmids are assigned to various types of incompatibility type.[7,12–18] IMP-1 family gene-containing class 1 integron has been detected on IncN, IncW, IncHI2, IncFIB and IncL/M plasmids in Enterobacterales.[9,17,19–23]

IncN plasmids belonging to plasmid ST 5 (IncN-pST5) have a conserved structure and sequence. The IncN-pST5 plasmids, which have been reported in several papers, are characterized by the common presence of both $bla_{CTX\text{-}M\text{-}2}$ and class 1 integron.[19,20,23,24] The integrons located in IncN-pST5 carry either $bla_{IMP\text{-}1}$ or $bla_{IMP\text{-}6}$. Both $bla_{CTX\text{-}M\text{-}2}$- and $bla_{IMP\text{-}1}$-carrying IncN-pST5 were detected in the *Klebsiella pneumoniae* that caused an outbreak at a hospital in Tokyo,[23] and both $bla_{CTX\text{-}M\text{-}2}$- and $bla_{IMP\text{-}6}$-carrying IncN-pST5 was detected in multiple species of Enterobacterales at a hospital in Osaka.[19] Based on the previously reported information, two hypotheses can be proposed for the formation of the two IncN-pST5 plasmids: either the evolution from $bla_{IMP\text{-}1}$ to $bla_{IMP\text{-}6}$ is due to point mutations, or it is the result of different genetic events, which may involve horizontal $bla_{IMP\text{-}1}$ or $bla_{IMP\text{-}6}$ transfer.

The aim of this study was to conduct a retrospective analysis of our bacterial strain collection, along with public database sequences, to investigate the formation process of IncN-pST5 plasmids. We aimed to specifically elucidate the stepwise acquisition of $bla_{CTX\text{-}M\text{-}2}$ by IncN-pST5, followed by the acquisition of either $bla_{IMP\text{-}1}$ or $bla_{IMP\text{-}6}$.

Materials and methods

Bacterial strains

A total of 281 bla_{IMP}- or $bla_{CTX\text{-}M}$-positive Enterobacterales strains had undergone draft WGS using the Illumina platform [Figure 1 and Table S1 (available as Supplementary data at *JAC-AMR* Online)]. These strains were isolated from human, chicken and broiler faeces between 1997 and 2019 in Japan. We performed a retrospective analysis on our strain collection to select both bla_{IMP}- and IncN-pST5-positive, as well as $bla_{CTX\text{-}M}$- and IncN-pST5-positive strains. From the collection, we selected strains that were positive for both $bla_{CTX\text{-}M}$ and IncN-pST5 ($n=37$) and both bla_{IMP} and IncN-pST5 ($n=83$). We focused on the IncN-pST5 plasmid from different *Escherichia coli* STs and performed long-read sequencing (Figure 1 and Table S2).

Draft WGS and analysis

DNA was extracted from cultured bacteria using a boil method and subsequently purified using Agencourt AMPure XP (Beckman Coulter, Brea, CA, USA). DNA libraries were prepared using the Illumina DNA Prep kit (Illumina, Inc., CA, USA). These DNA libraries were sequenced using MiSeq (Illumina) with 300 bp paired-end reads or NovaSeq 6000 (Illumina) with 150 bp paired-end reads. The sequencing data were trimmed to remove adapter sequences and were required to maintain a quality score higher than Q30 using Trimmomatic version 0.39,[25] followed by *de novo* assembly using SPAdes 3.15.2.[26]

Species identification was performed using average nucleotide identity (ANI), with a cut-off value of ≥96%.[27] These were compared with the genomic sequences of the type strain, which were downloaded and referenced from the NCBI Taxonomy database. MLST, identification of acquired antimicrobial resistance genes, detection of antimicrobial-resistant

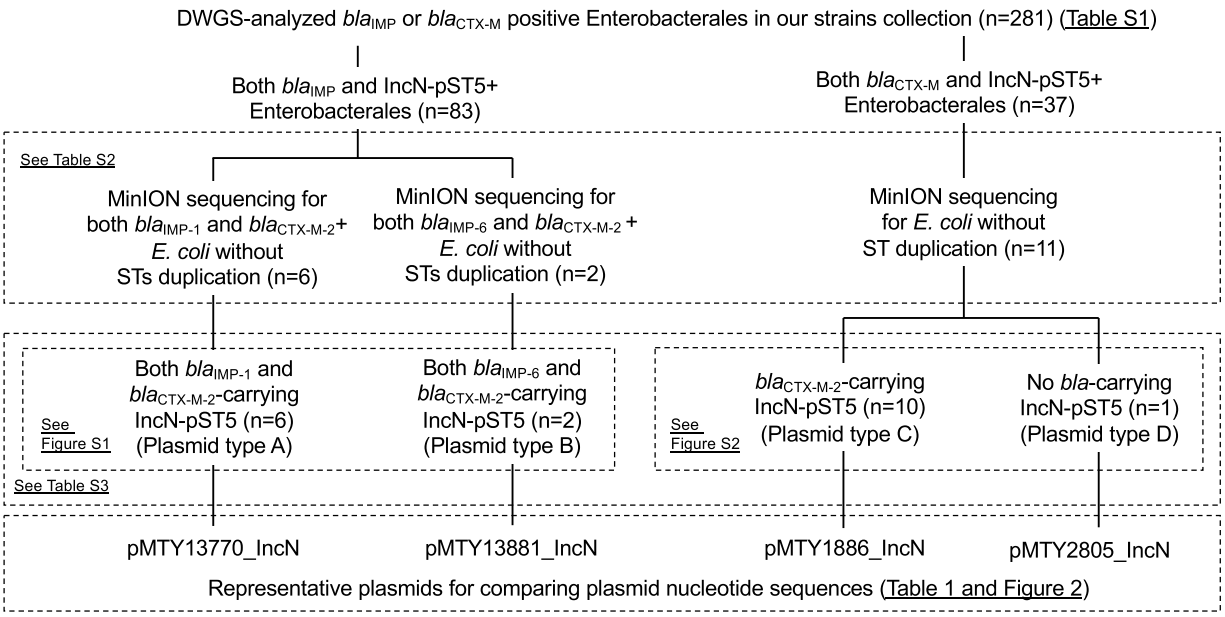

Figure 1. The strain selection process for comparative analysis of IncN-pST5 plasmid used in this study. We utilized $bla_{CTX\text{-}M}$- and bla_{IMP}-positive strains ($n=281$) from our strain collection. These strains had undergone draft WGS using MiSeq (Illumina), but the results remain unpublished (Table S1). Strains positive for both $bla_{CTX\text{-}M}$ and IncN-pST5, as well as strains positive for both bla_{IMP} and IncN-pST5, are listed in Table S2. Different ST strains were selected and sequenced using MinION for comparison of plasmid structure. Sequence comparison of IncN-pST5 carrying both $bla_{CTX\text{-}M\text{-}2}$ and either $bla_{IMP\text{-}1}$ or $bla_{IMP\text{-}6}$ in our collection and IncN-pST5 not carrying $bla_{IMP\text{-}1}$ or $bla_{IMP\text{-}6}$ in our collection are shown in Figure S1 and Figure S2, respectively. Four representative types of plasmid structure are shown in Figure 2.

determinant mutations, and recognition of plasmid replicon type were performed using MLST version 2.0, ResFinder version 4.1, PointFinder version 2.1 and PlasmidFinder version 2.1, respectively. We ran those programs with default parameters. These resources are available at the Center for Genomic Epidemiology (http://genomicepidemiology.org/).

Long-read sequencing and plasmid comparisons

DNA extraction was performed using the magLEAD 6gC (Precision System Science Co., Ltd., Matsudo, Japan) following the MagDEA Dx SV PS protocol. DNA libraries were prepared using the Rapid Barcoding Kit SQK-RBK004 (Oxford Nanopore Technologies: ONT, Oxford, UK). Sequencing was carried out using the MinION (ONT) fitted with MinION flow cell R9.4 (ONT). Basecalling and demultiplexing were executed by Guppy v5.0.11 (ONT). MinION reads were assembled with Illumina sequencer read data using Unicycler (version 0.4.8-beta). Finally, contig polishing was performed three times using Pilon.

Plasmid analysis

Sequence data were annotated using the DNA Data Bank of Japan (DDBJ) Fast Annotation and Submission Tool (DFAST). ISs were confirmed using ISFinder (https://isfinder.biotoul.fr/). Comparisons of plasmid structures were performed using Easyfig 2.2.2. Integron number (In) was assigned using the INTEGRALL database (http://integrall.bio.ua.pt/?).[28]

Conjugal transfer experiments

Conjugal transfer experiments were performed using the filter-mating method. The donor isolates employed in this study were *E. coli* strains harbouring the IncN-pST5 plasmid. A rifampicin-resistant, lactose-non-fermenting *E. coli* strain (ML4909) was used as a recipient.[29] Transconjugants were selected on MacConkey agar plates (Eiken Chemical Co., Ltd., Tokyo, Japan) containing both cefotaxime (4 mg/L, Sigma–Aldrich) and rifampicin (100 mg/L, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). The presence of IncN plasmid carrying $bla_{CTX\text{-}M\text{-}2}$ in transconjugants was confirmed by PCR. The frequency of conjugation was calculated by dividing the number of transconjugants (cfu/mL) by the number of recipient bacteria.

Data availability

The WGS data have been deposited in GenBank under the BioProject accession numbers PRJDB13833, PRJNA980932, PRJNA984319, PRJNA984451 and PRJNA984747. The specific accession numbers for the WGS data of each strain and plasmid can be found in Tables S1 and S2.

Results

WGS of IncN-pST5 harbouring strains

We focused on IncN-pST5 and the genes it carries, bla_{IMP} or $bla_{CTX\text{-}M}$, in this study. Initially, we identified 281 bla_{IMP}- or $bla_{CTX\text{-}M}$-positive strains from our strain collection, which had undergone draft WGS (Figure 1 and Table S1). Second, we selected 83 strains that were both bla_{IMP} and IncN-pST5 positive and 37 strains that were $bla_{CTX\text{-}M}$ and IncN-pST5 positive (Figure 1). To avoid analysing identical or extremely closely related strains that might have spread during transmission in hospitals or in poultry processing, we selected *E. coli* strains from different STs. These included six strains that were both $bla_{IMP\text{-}1}$ and IncN-pST5 positive, two strains that were both $bla_{IMP\text{-}6}$ and $bla_{CTX\text{-}M\text{-}2}$ positive, and 11 strains that were both $bla_{CTX\text{-}M}$ and IncN-pST5 positive (Figure 1).

Complete sequence of IncN-pST5

Hybrid *de novo* assembly using MiSeq and MinION data revealed circular IncN-pST5 plasmids in 6 strains carrying both $bla_{IMP\text{-}1}$ and $bla_{CTX\text{-}M\text{-}2}$, 2 strains carrying both $bla_{IMP\text{-}6}$ and $bla_{CTX\text{-}M\text{-}2}$, 10 strains carrying $bla_{CTX\text{-}M\text{-}2}$, and 1 strain not carrying any *bla* genes (Figures 1 and 2). The average sequence depth was 197× (SD, 141×). The backbone structure of IncN-pST5 plasmids from different *E. coli* STs was found to be highly conserved (99.90%) (Figures S1 and S2 and Table S2). An exception was pMTY14771_IncN-X1, which was constructed as a multi-replicon with the IncX1 plasmid.

IncN-pST5 categorization and structural comparison

The plasmids were categorized into four plasmid types based on the β-lactamase genes they carried: type A, which carries both $bla_{IMP\text{-}1}$ and $bla_{CTX\text{-}M\text{-}2}$ ($n=6$); type B, which carries both $bla_{IMP\text{-}6}$ and $bla_{CTX\text{-}M\text{-}2}$ ($n=2$); type C, which carries $bla_{CTX\text{-}M\text{-}2}$ ($n=10$); and type D, which carries no β-lactamase genes ($n=1$) (Table 1 and Table S2 and Figure 2). The type D plasmid was harboured by *E. coli* TUM2805, which had a $bla_{CTX\text{-}M\text{-}14}$-carrying IncB/O/K/Z plasmid. Representative plasmids from each type (pMTY13770_IncN for type A, pMTY13881_IncN for type B, pMTY1886_IncN for type C, and pMTY2805_IncN for type D) were similar except for the region carrying the β-lactamase gene, such as the class 1 integron and the IS*Ecp1*-$bla_{CTX\text{-}M\text{-}2}$ element. Type A had a 3414 bp inversion containing *tet*(A).

β-Lactamase genes and MGEs on IncN-pST5 plasmids

Plasmid types A, B and C were found to carry $bla_{CTX\text{-}M\text{-}2}$, which is mediated by IS*Ecp1*, in the same position (Figure 2). Additionally, $bla_{IMP\text{-}1}$ in plasmid type A and $bla_{IMP\text{-}6}$ in plasmid type B were identified in class 1 integrons In798 and In1690, respectively. The locations of class 1 integrons in plasmid types A and B were similar (Figure 2). We also compared the structure of the plasmids between types A and B with those previously reported. Plasmids that exhibited more than 90% similarity based on nucleotide BLAST against GenBank were found to be structurally conserved (Figures S3 and S4 and Tables S3 and S4). Furthermore, we discovered that the overall structure of plasmid type B is very similar to that of plasmids forming hybrids with other replicon types (IncFII, FIA, FIB or IncR) (Figure S4 and Table S4). A structural comparison of pKP96, which belongs to IncN-pST5 and is larger in size than type A, revealed that the position of the backbone and class 1 integron (In62) were conserved (Figure S5).

Conjugation of IncN-pST5 plasmids

All strains carrying representative plasmid types A to C were conjugatively transferred to the recipient strain. The transfer frequencies were as follows: for plasmid type A (pMTY13770_IncN), 5.2×10^{-1}; for plasmid type B (pMTY13881_IncN), 1.3×10^{-1}; and for plasmid type C (pMTY1886_IncN), 2.5×10^{-2}.

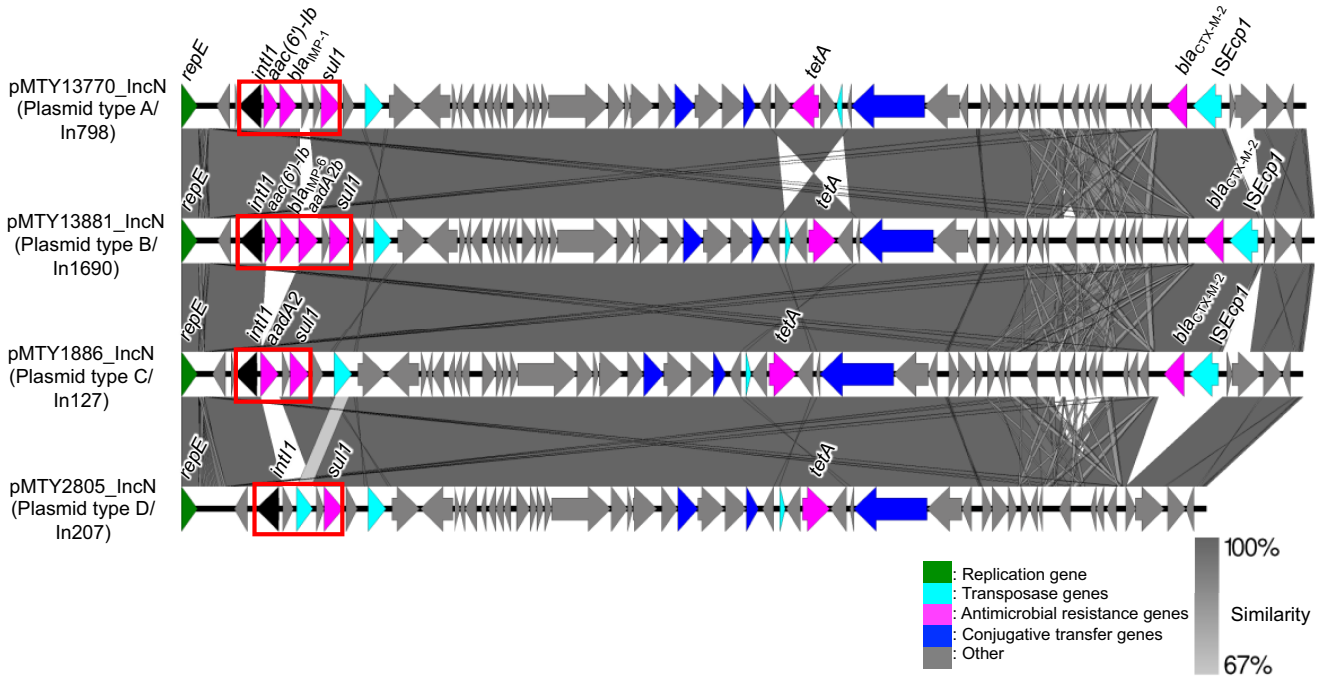

Figure 2. Comparison of four representative types of IncN-pST5 plasmid and class 1 integron structure. The circular plasmid structure is represented linearly. The integron number and plasmid types are shown alongside the plasmid name. The arrow indicates the ORF.

Table 1. Characteristics of IncN-pST5 plasmids

Plasmid type	Representative plasmid name	Antimicrobial resistance genes carried	Class 1 integron	Plasmid length (bp)	Host species	Isolation year	GenBank accession number
A	pMTY13770_IncN	*aac(6′)-Ib*, bla_{IMP-1}, *sul1*, *tet*(A), $bla_{CTX-M-2}$	In798	49 758	*E. coli*	2011	LC720959
B	pMTY13881_IncN	*aac(6′)-Ib*, bla_{IMP-6}, *aadA2b*, *sul1*, *tet*(A), $bla_{CTX-M-2}$	In1690	50 135	*E. coli*	2013	LC720960
C	pMTY1886_IncN	*aadA2*, *sul1*, *tet*(A), $bla_{CTX-M-2}$	In127	49 620	*E. coli*	2003	AP026458
D	pMTY2805_IncN	*sul1*, *tet*(A)	In207	45 363	*E. coli*	2006	AP026533

Discussion

We identified a probable ancestor of IncN-pST5 through WGS of our bacterial collection. The results suggested that the class 1 integron played a pivotal role in allowing IncN-pST5 to acquire the carbapenemase gene, either bla_{IMP-1} or bla_{IMP-6} (Figure 3). IncN-pST5 with HGT capability and both a class 1 integron and a conserved structure may be more likely to facilitate the widespread and long-term dissemination of the carbapenemase gene. The high conjugative frequency of IncN-pST5 was a significant contributing factor to the outbreak of IMP-1- or IMP-6-producing multiple species and various genetic clades in Japan associated with the IncN-pST5 plasmid.[19,23]

Gene gain/loss on the plasmid spans a large region involving ISs, Tns and Ins.[30,31] Even with WGS, understanding the order of construction for recently detected plasmids through antimicrobial resistance gene acquisition can be challenging, depending on the plasmid structure. Another plasmid, known as IncW, could serve as a helpful reference for understanding IncN-pST5. This is because IncW has several features in common with IncN: it is genetically close to IncN based on the *mob* sequence, its backbone structure is highly conserved, and it harbours a class 1 integron.[31] The plasmid structure of bla_{IMP-1}-carrying IncW pMTY10660_IncW, detected in 2010, resembles pR388 carrying a class 1 integron without bla_{IMP-1}, detected in the 1970s.[17,32] We have identified the sequence of IncN-pST5 pMTY2805_IncN, carrying a class 1 integron without bla_{IMP} from *E. coli* isolated in 2006 (Table 1 and Figure 2). This is similar to the story of IncW acquiring bla_{IMP-1}, suggesting that IncN-pST5, like IncW, originally carried a class 1 integron, which was important for bla_{IMP-1} acquisition. To date, bla_{IMP-6} has not been detected in any plasmid other than IncN. If frequent point

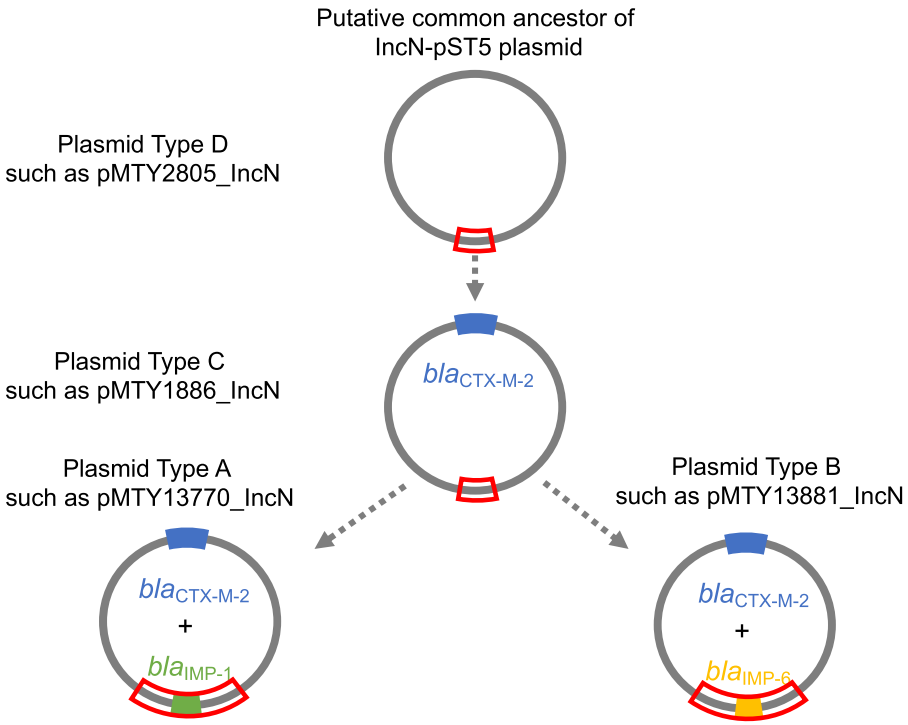

Figure 3. Likely molecular processes of *bla*$_{CTX-M-2}$ and *bla*$_{IMP-1}$ or *bla*$_{IMP-6}$ acquisition in IncN-pST5 plasmids. The red box indicates class 1 integron. Plasmid type D is a common ancestor of IncN-pST5. Plasmid types A and B have separated from type C after acquiring *bla*$_{CTX-M-2}$.

mutations from *bla*$_{IMP-1}$ to *bla*$_{IMP-6}$ occur, then we should be able to find a plasmid carrying *bla*$_{IMP-6}$ other than IncN.

pMTY2805_IncN is likely an ancestor of IncN-pST5 because the position of the IS*Ecp1*-*bla*$_{CTX-M-24}$ element on pKP96, which belongs to IncN-pST5, differs from that on pMTY1886_IncN (Figure S5 and Figure 2). The difference in the position of the IS*Ecp1*-*bla*$_{CTX-M}$ element between pKP96 and the sequenced plasmids, pMTY1886_IncN, pMTY13770_IncN and pMTY13881_IncN, suggested that they diverged from a pMTY2805_IncN-like plasmid. Subsequently, pMTY13770_IncN and pMTY13881_IncN appear to have been derived from a pMTY1886_IncN-like plasmid independently because there was an inversion around *tet*(A) and a deletion upstream of IS*Ecp1* and *bla*$_{CTX-M}$.

There are three limitations in this study. First, we conducted a retrospective study using strains available in our laboratory (Figure 1). Furthermore, we excluded strains that appeared to be involved in nosocomial transmission based on associated information such as the hospital of isolation, period and source. Second, we included unpublished WGS data from other projects in our genome analysis and provided limited sample metadata. Third, based on representative plasmid sequence data, we estimated the most likely path of genetic acquisition for *bla*$_{CTX-M-2}$ and the subsequent acquisition of either *bla*$_{IMP-1}$ or *bla*$_{IMP-6}$ during the construction of IncN-pST5.

In this study, we demonstrated that IncN-pST5 acquired *bla*$_{CTX-M-2}$, along with either *bla*$_{IMP-1}$ or *bla*$_{IMP-6}$, in a stepwise manner. IncN-pST5 inherently possesses a class 1 integron, which may have predisposed it to *bla*$_{IMP}$ acquisition. The fact that the class 1 integron is carried on a plasmid with extremely high conjugative transfer efficiency, such as IncN-pST5, highlights the risk of antimicrobial resistance genes spreading through HGT.

Acknowledgements

We thank Dr Tomoko Uehira of the Department of Infectious Diseases, National Hospital Organization Osaka National Hospital, Osaka, Japan, and Dr Yuho Horikoshi of the Division of Infectious Diseases, Tokyo Metropolitan Children's Medical Center, Tokyo, Japan, for providing the strains to our collection.

Funding

This work was supported by the Ministry of Health, Labour, and Welfare of Japan (grant number: H30-Shokuhin-Ippan-006 and 21KA1004) and the Japan Society for the Promotion of Science KAKENHI Grant-in-Aid for Scientific Research(C) to Y.I. (grant number: JP18K08452).

Transparency declarations

All authors: none to declare.

Supplementary data

Figures S1 to S5 and Tables S1 to S4 are available as Supplementary data at *JAC-AMR* Online.

References

1 Kopotsa K, Osei Sekyere J, Mbelle NM. Plasmid evolution in carbapenemase-producing Enterobacteriaceae: a review. *Ann N Y Acad Sci* 2019; **1457**: 61–91. https://doi.org/10.1111/nyas.14223

2 Khedkar S, Smyshlyaev G, Letunic I *et al*. Landscape of mobile genetic elements and their antibiotic resistance cargo in prokaryotic genomes. *Nucleic Acids Res* 2022; **50**: 3155–68. https://doi.org/10.1093/nar/gkac163

3 Cantón R, González-Alba JM, Galán JC. CTX-M enzymes: origin and diffusion. *Front Microbiol* 2012; **3**: 110. https://doi.org/10.3389/fmicb.2012.00110

4 Karim A, Poirel L, Nagarajan S *et al*. Plasmid-mediated extended-spectrum β-lactamase (CTX-M-3 like) from India and gene association with insertion sequence IS*Ecp1*. *FEMS Microbiol Lett* 2001; **201**: 237–41. https://doi.org/10.1111/j.1574-6968.2001.tb10762.x

5 Hamamoto K, Tokunaga T, Yagi N *et al*. Characterization of $bla_{CTX-M-14}$ transposition from plasmid to chromosome in *Escherichia coli* experimental strain. *Int J Med Microbiol* 2020; **310**: 151395. https://doi.org/10.1016/j.ijmm.2020.151395

6 Partridge SR, Tsafnat G, Coiera E *et al*. Gene cassettes and cassette arrays in mobile resistance integrons. *FEMS Microbiol Rev* 2009; **33**: 757–84. https://doi.org/10.1111/j.1574-6976.2009.00175.x

7 Matsumura Y, Peirano G, Motyl MR *et al*. Global molecular epidemiology of IMP-producing Enterobacteriaceae. *Antimicrob Agents Chemother* 2017; **61**: e02729-16. https://doi.org/10.1128/AAC.02729-16

8 Papagiannitsis CC, Dolejska M, Izdebski R *et al*. Characterization of pKP-M1144, a novel ColE1-like plasmid encoding IMP-8, GES-5, and BEL-1 β-lactamases, from a *Klebsiella pneumoniae* sequence type 252 isolate. *Antimicrob Agents Chemother* 2015; **59**: 5065–8. https://doi.org/10.1128/AAC.00937-15

9 Zhao W-H, Hu Z-Q. IMP-type metallo-β-lactamases in Gram-negative bacilli: distribution, phylogeny, and association with integrons. *Crit Rev Microbiol* 2011; **37**: 214–26. https://doi.org/10.3109/1040841X.2011.559944

10 Walsh TR, Toleman MA, Poirel L *et al*. Metallo-β-lactamases: the quiet before the storm? *Clin Microbiol Rev* 2005; **18**: 306–25. https://doi.org/10.1128/CMR.18.2.306-325.2005

11 Zhao W-H, Hu Z-Q. Epidemiology and genetics of VIM-type metallo-β-lactamases in Gram-negative bacilli. *Future Microbiol* 2011; **6**: 317–33. https://doi.org/10.2217/fmb.11.13

12 García-Fernández A, Villa L, Moodley A *et al*. Multilocus sequence typing of IncN plasmids. *J Antimicrob Chemother* 2011; **66**: 1987–91. https://doi.org/10.1093/jac/dkr225

13 Partridge SR. Tn*4401* carrying bla_{KPC} is inserted within another insertion in pKpQIL and related plasmids. *J Clin Microbiol* 4401; **2014**: 4448–9. https://doi.org/10.1128/JCM.02426-14

14 Li W, Guo H, Gao Y *et al*. Comparative genomic analysis of plasmids harboring bla_{OXA-48}-like genes in *Klebsiella pneumoniae*. *Front Cell Infect Microbiol* 2022; **12**: 1082813. https://doi.org/10.3389/fcimb.2022.1082813

15 Wailan AM, Sidjabat HE, Yam WK *et al*. Mechanisms involved in acquisition of bla_{NDM} genes by IncA/C2 and IncFIIY plasmids. *Antimicrob Agents Chemother* 2016; **60**: 4082–8. https://doi.org/10.1128/AAC.00368-16

16 Wu W, Feng Y, Tang G *et al*. NDM metallo-β-lactamases and their bacterial producers in health care settings. *Clin Microbiol Rev* 2019; **32**: e00115-18. https://doi.org/10.1128/CMR.00115-18

17 Aoki K, Harada S, Yahara K *et al*. Molecular characterization of IMP-1-producing *Enterobacter cloacae* complex isolates in Tokyo. *Antimicrob Agents Chemother* 2018; **62**: e02091-17. https://doi.org/10.1128/AAC.02091-17

18 Botelho J, Grosso F, Quinteira S *et al*. Two decades of bla_{VIM-2}-producing *Pseudomonas aeruginosa* dissemination: an interplay between mobile genetic elements and successful clones. *J Antimicrob Chemother* 2018; **73**: 873–82. https://doi.org/10.1093/jac/dkx517

19 Yamagishi T, Matsui M, Sekizuka T *et al*. A prolonged multispecies outbreak of IMP-6 carbapenemase-producing Enterobacterales due to horizontal transmission of the IncN plasmid. *Sci Rep* 2020; **10**: 4139. https://doi.org/10.1038/s41598-020-60659-2

20 Kayama S, Shigemoto N, Kuwahara R *et al*. Complete nucleotide sequence of the IncN plasmid encoding IMP-6 and CTX-M-2 from emerging carbapenem-resistant Enterobacteriaceae in Japan. *Antimicrob Agents Chemother* 2015; **59**: 1356–9. https://doi.org/10.1128/AAC.04759-14

21 Mori N, Tada T, Oshiro S *et al*. A transferrable IncL/M plasmid harboring a gene encoding IMP-1 metallo-β-lactamase in clinical isolates of Enterobacteriaceae. *BMC Infect Dis* 2021; **21**: 1061. https://doi.org/10.1186/s12879-021-06758-5

22 Kubota H, Suzuki Y, Okuno R *et al*. IMP-68, a novel IMP-type metallo-β-lactamase in imipenem-susceptible *Klebsiella pneumoniae*. *mSphere* 2019; **4**: e00736-19. https://doi.org/10.1128/mSphere.00736-19

23 Ito K, Honda H, Yoshida M *et al*. A metallo-beta-lactamase producing Enterobacteriaceae outbreak from a contaminated tea dispenser at a children's hospital in Japan. *Infect Control Hosp Epidemiol* 2019; **40**: 217–20. https://doi.org/10.1017/ice.2018.331

24 Endo YT, Aoki K, Hamada M *et al*. Full-length whole-genome sequencing analysis of emerged meropenem-resistant mutants during long-term *in vitro* exposure to meropenem for borderline meropenem-susceptible carbapenemase-producing and non-carbapenemase-producing Enterobacterales. *J Antimicrob Chemother* 2022; **78**: 209–15. https://doi.org/10.1093/jac/dkac376

25 Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 2014; **30**: 2114–20. https://doi.org/10.1093/bioinformatics/btu170

26 Bankevich A, Nurk S, Antipov D *et al*. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J Comput Biol* 2012; **19**: 455–77. https://doi.org/10.1089/cmb.2012.0021

27 Kim M, Oh H-S, Park S-C *et al*. Towards a taxonomic coherence between average nucleotide identity and 16S rRNA gene sequence similarity for species demarcation of prokaryotes. *Int J Syst Evol Microbiol* 2014; **64**: 346–51. https://doi.org/10.1099/ijs.0.059774-0

28 Moura A, Soares M, Pereira C *et al*. INTEGRALL: a database and search engine for integrons, integrases and gene cassettes. *Bioinformatics* 2009; **25**: 1096–8. https://doi.org/10.1093/bioinformatics/btp105

29 Ma L, Ishii Y, Ishiguro M *et al*. Cloning and sequencing of the gene encoding toho-2, a class A β-lactamase preferentially inhibited by tazobactam. *Antimicrob Agents Chemother* 1998; **42**: 1181–6. https://doi.org/10.1128/AAC.42.5.1181

30 Partridge SR, Kwong SM, Firth N *et al*. Mobile genetic elements associated with antimicrobial resistance. *Clin Microbiol Rev* 2018; **31**: e00088-17. https://doi.org/10.1128/CMR.00088-17

31 Rozwandowicz M, Brouwer MSM, Fischer J *et al*. Plasmids carrying antimicrobial resistance genes in Enterobacteriaceae. *J Antimicrob Chemother* 2018; **73**: 1121–37. https://doi.org/10.1093/jac/dkx488

32 Paterson ES, Moré MI, Pillay G *et al*. Genetic analysis of the mobilization and leading regions of the IncN plasmids pKM101 and pCU1. *J Bacteriol* 1999; **181**: 2572–83. https://doi.org/10.1128/JB.181.8.2572-2583.1999