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IncX2 and IncX1-X2 Hybrid Plasmids Coexisting in a FosA6-Producing *Escherichia coli* Strain

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ABSTRACT IncX plasmids are receiving much attention as vehicles of carbapenem and colistin resistance genes, such as bla_{NDM}, bla_{KPC}, and *mcr-1*. Among them, IncX2 subgroup plasmids remain rare. Here, we characterized IncX2 and IncX1-X2 hybrid plasmids coexisting in a FosA6-producing *Escherichia coli* strain that were possibly generated as a consequence of recombination events between an R6K-like IncX2 plasmid and a pLN126_33-like IncX1 plasmid. Variable multidrug resistance mosaic regions were observed in these plasmids, indicating their potential to serve as flexible carriers of resistance genes. The diversity of IncX group plasmid backbones and accessory genes and the evolution of hybrid IncX plasmids pose a challenge in detecting and classifying them.

KEYWORDS IncX plasmid, *taxC*, *pir*, genotyping, glutathione synthetase

Received 13 March 2017 **Returned for modification** 10 April 2017 **Accepted** 19 April 2017

Accepted manuscript posted online 24 April 2017

Citation Guo Q, Su J, McElheny CL, Stoesser N, Doi Y, Wang M. 2017. IncX2 and IncX1-X2 hybrid plasmids coexisting in a FosA6-producing *Escherichia coli* strain. Antimicrob Agents Chemother 61:e00536-17. https://doi.org/10.1128/AAC.00536-17.

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The IncX family plasmids are narrow-host-range plasmids mostly found in *Enterobacteriaceae* (1–3). IncX plasmids contain a highly syntenic backbone consisting of core genes responsible for plasmid replication, partitioning, maintenance, and conjugal DNA transfer (2). So far, at least eight subgroups of IncX plasmids have been reported, IncX1 to IncX8, carrying various resistance genes, including *oqxAB*, *qnrS1*, *qnrS2*, bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{KPC}, bla_{NDM}, and *mcr-1*, and mediating resistance to fluoroquinolones, cephalosporins, carbapenems, colistin, or other types of antibiotics (2–9). Plasmid R6K, recovered from an *Escherichia coli* strain cultured in Greece in the 1960s, is considered to be the prototypical IncX plasmid (1). R6K belongs to a rare IncX subgroup, IncX2, with only two other currently recognized members, pNGX2-QnrS1 (GenBank accession number JQ269335.1) and pEBG1 (GenBank accession number KF738053.1), both discovered in strains from western Nigeria and carrying *qnrS1* genes (3, 10). In contrast, the IncX1, IncX3, and IncX4 subgroup plasmids are much more prevalent and are associated with *oqxAB*, bla_{NDM}, and *mcr-1*, respectively (2, 3, 7, 8, 11). Here, we describe a new IncX2 plasmid, pYD786-4, that coexisted with a hybrid IncX1-X2 plasmid pYD786-3 in an *E. coli* ST410 urinary tract strain collected from a patient in the United States (12).

RESULTS AND DISCUSSION

Hybrid IncX1-X2 plasmid pYD786-3. Plasmid pYD786-3 is 44,806 bp in size and shares conserved backbone modules of both IncX1 and IncX2 plasmids. The replication, partitioning, and stability regions are from the IncX1 plasmid, while the maintenance and DNA transfer regions are from the IncX2 plasmid (Fig. 1). *pir* and *bis* encode the plasmid initiation replication protein and auxiliary protein that bind to the core region of replication gamma origin (2). *parGF* and *stbED* have putative functions of partitioning and stability. Two long inverted repeats (LIRs), α-LIR and β-LIR, contain origins of

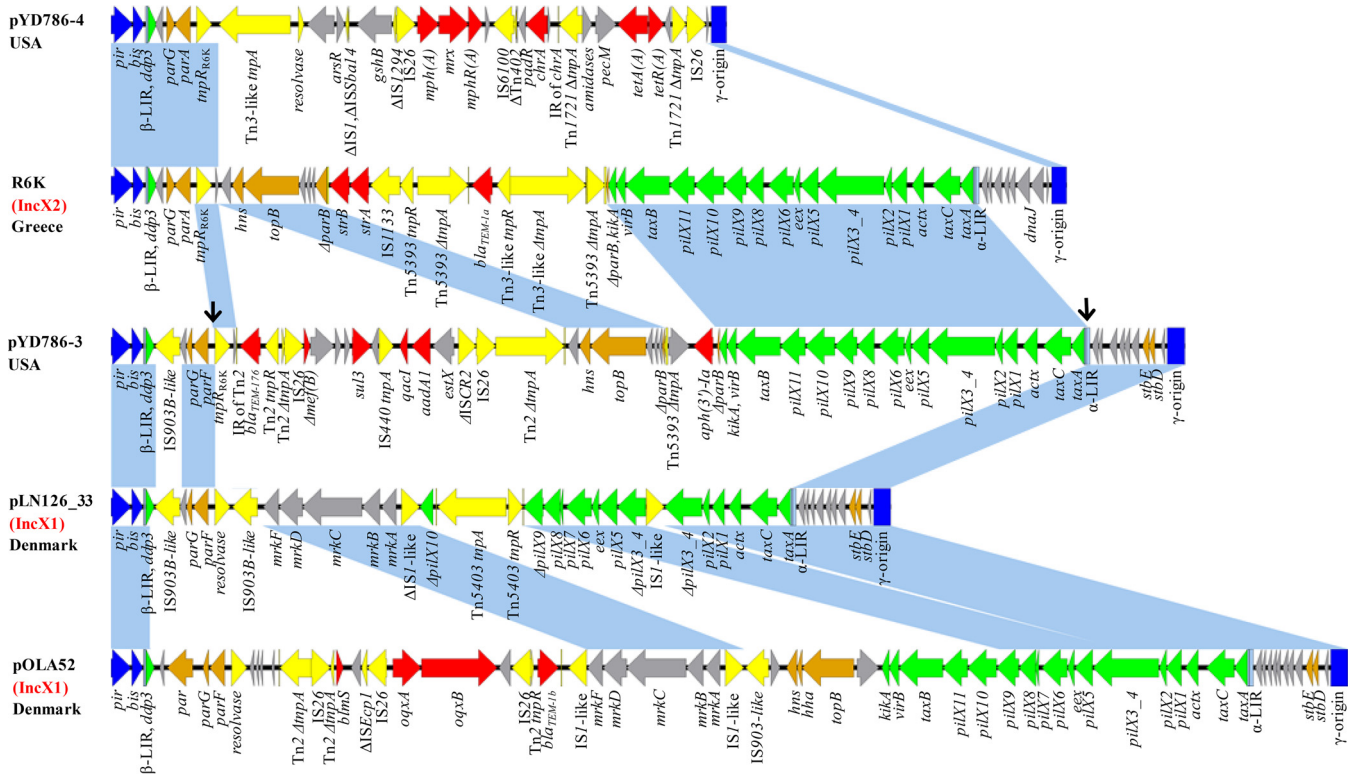

FIG 1 Linear comparison of rearranged IncX1 and IncX2 plasmids including pYD786-3 (GenBank accession number KU254580.1), pYD786-4 (KU254581.1), pLN126_33 (HE578058.1), pOLA52 (EU370913.1), and R6K (NCTC50005 [http://www.sanger.ac.uk/resources/downloads/plasmids/]). Genes are colored based on the predicted function: blue, replication; brown, partition, stability, and maintenance; green, DNA transfer; yellow, mobile genetic elements; red, resistance; light gray, other functions. Black arrows indicate putative recombination junction sites in pYD786-3. Shading between plasmids indicates shared backbone regions with a nucleotide identity of >99.3% to 100%.

conjugal transfer (*oriT*) which act with DNA distortion proteins (DDP) TaxC and TaxA to initiate the relaxation and nicking of *oriT* (2, 13). There are also genes (*hns* and *topB*) encoding putative maintenance functions and genes (*kikA*, *pilX*, *actX*, and *taxBCA*) encoding conjugative DNA transfer in pYD786-3. An 8,411-bp fragment organized as α-LIR (in part)–*stbED*–gamma origin–*pir*–*bis*–β-LIR–*ddp3*–*parGF* is almost identical to the core genes of IncX1 plasmid pLN126_33 (GenBank accession number HE578058.1) (14), except for two point mutations, two indels (insertions and deletions), and insertion of an IS*903B*-like sequence (Fig. 1). Plasmid pLN126_33 is structurally similar to pOLA52 (GenBank accession number EU370913.1), a representative and well-characterized IncX1 plasmid carrying the type 3 fimbria gene cassette *mrkABCDF* (2, 14). In contrast, the rest of pYD786-3 (36,395 bp long), including regions of *hns-topB* and *kikA-taxB-pilX-actX-taxCA*, differs from the IncX2 plasmid R6K by only three single nucleotides and the presence of two accessory modules composed of mobile genetic elements (MGEs) and antimicrobial resistance genes (Fig. 1). Interestingly, pYD786-4, a small plasmid of 25,678 bp, possesses the 5.3-kb backbone of R6K containing gamma origin–*pir*–*bis*–β-LIR–*ddp3*–*parGA*, with only one nucleotide difference and one indel. Therefore, pYD786-3 and pYD786-4 were probably generated as a consequence of recombinations between an R6K-like IncX2 plasmid and a pLN126_33-like IncX1 plasmid in *E. coli* YD786.

Two putative recombination junction sites are indicated in Fig. 1 (see also Fig. S1 in the supplemental material), one located between *parA* and the *tnpR* gene from R6K ($tnpR_{R6K}$) (Fig. S1A) and the other in α-LIR (Fig. S1B), thus creating two hybrid IncX plasmids. pYD786-3 is characterized by a mosaic IncX1-X2 backbone, a hybrid α-LIR, and a resolvase gene, $tnpR_{R6K}$, from R6K (Fig. 1). Wider BLASTn comparisons with publicly available plasmids showed that plasmid C (GenBank accession number CP010155.1) in *E. coli* strain D9 possessed a backbone almost identical to that of

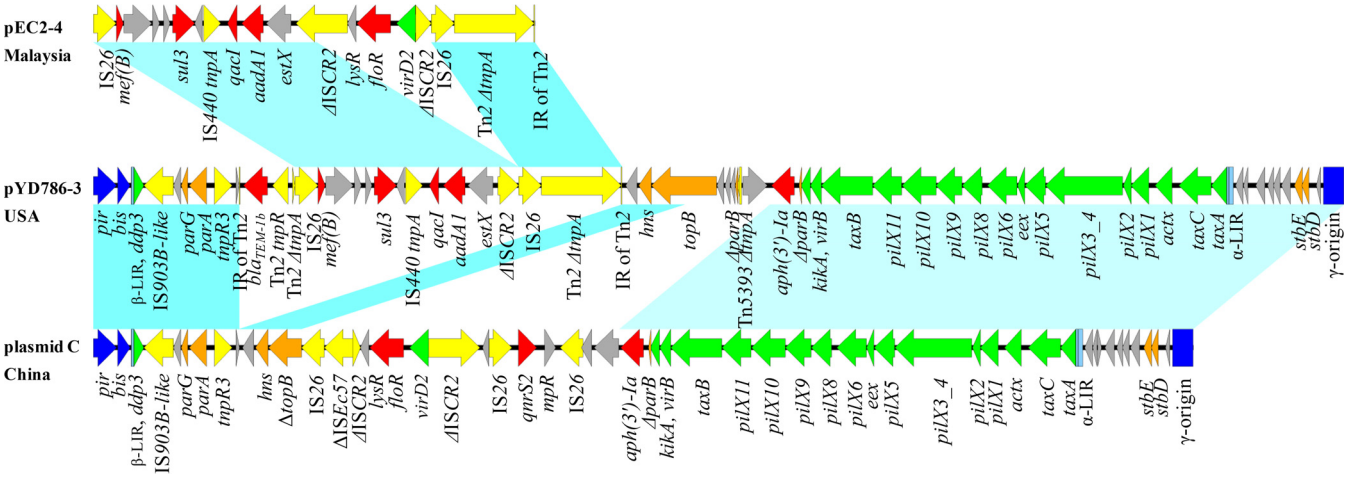

FIG 2 Comparison of backbone and multidrug resistance regions of rearranged plasmid pYD786-3 (GenBank accession number KU254580.1) with those of plasmid C (CP010155.1; rearranged and inversely displayed) and pEC2-4 (CP016184.1; nucleotides 23922 to 39746). Shading between plasmids indicates shared backbone regions with a nucleotide identity of >99.9% to 100%.

pYD786-3, with only five nucleotide differences (Fig. 2). A similar structure was also found in the chromosome of *E. coli* strain D4 (GenBank accession number CP010143.1); both strains were isolated from Chinese dogs. Interestingly, $tnpR_{R6K}$, belonging to the serine recombinase family, is specifically located in IncX2 plasmids (R6K, pNGX2-QnrS1, pEBG1, and pYD786-4) or their hybrid derivatives (pYD786-3, plasmid C, and *E. coli* strain D4), indicating its ancient linkage with this subgroup of plasmids and global dissemination.

Most recently, an IncX4-X3 hybrid plasmid, pCQ02-121 (GenBank accession number KU647721.1), was discovered in an *E. coli* strain from a cat in China harboring bla_{NDM-5} and *mcr-1* resistance determinants (7). Similar to pYD786-3, pCQ02-121 contained replication and partition regions from a pMCR1_IncX4-like plasmid and DNA transfer and maintenance regions (*hns-topB-pilX-taxBCA*) from a pNDM5_IncX3-like plasmid (7). Plasmids pNDM5_IncX3 (GenBank accession no. KU761328.1) and pMCR1_IncX4 (KU761327.1) were reported to coexist in the same clinical strain of *Klebsiella pneumoniae* in China (15), providing another piece of evidence for *in vivo* rearrangements of IncX plasmids.

The MRR of pYD786-3. A Tn*2* transposon, bearing the *β*-lactamase gene $bla_{TEM-176}$, is inserted into the backbone of pYD786-3, followed by recruitment of an IS*26*-flanked composite transposon harboring an uncommon *sul3*-type integron and interrupting the *tnpA* of Tn*2* (16). The *sul3*-type integron carries gene cassettes *estX-aadA1-qacI*, followed by IS*440-sul3-*Δ*mefB* rather than the 3′ conserved segment (CS). The 5′ CS is replaced by a truncated IS*CR2*, thus generating a multidrug resistance mosaic region (MRR) in pYD786-3. Almost identical MRRs have been identified in two *mcr-1*-bearing plasmids of *E. coli*, isolated from Malaysian food animals (pEC2_1-4, GenBank accession number CP016183.1; pEC2-4, GenBank accession number CP016184.1) (Fig. 2), indicating IS*26*-mediated mobilization of the fragment Δ*ISCR2-estX-aadA1-qacI*-IS*440-sul3-*Δ*mefB* among strains though the combined unit has rarely been reported. The other variable region of pYD786-3 is composed of a truncated Tn*5393* and an aminoglycoside resistance gene, *aph*(*3′*)*-Ia*, located between *hns-topB* and the DNA transfer region.

pYD786-4, a new IncX2 plasmid. Unlike classic IncX2 plasmids, pYD786-4 has lost part of its core DNA transfer and mating pair formation system (*pilX* and *taxCAB*) genes, possibly due to the recombination between two IncX plasmids and subsequent recruitment of resistance genes with MGEs. The MRR of pYD786-4 is composed of a Tn*3*-like transposon, an IS*26*-flanked composite transposon, and a glutathione synthetase gene (*gshB*) flanked by truncated IS*1294*, IS*Sba14*, and IS*1* (Fig. 1). Tn*1721*, carrying *tetR*(A) and *tetA*(A), is truncated by IS*26* and an inverted repeat carrying the *chrA* gene

(IR_{chrA})-*chrA* module, followed by IRt-IS*6100* (where IRt is an inverted repeat of class 1 integrons) and a macrolide resistance module bound at one end by another IS*26*, as described previously (17). Since glutathione is a substrate of the glutathione *S*-transferase FosA6, which inactivates fosfomycin by forming a glutathione-fosfomycin adduct (12), the glutathione synthetase here may play a role in modulating the function of FosA6. The *gshB* genes have been found on 13 plasmids and one chromosome, with 9 of them coexisting with the fosfomycin resistance gene *fosA3* or *fosA6* (Table S1). The *gshB* genes are carried by *E. coli* or *Salmonella* species isolated from humans, animals, forest soil, or sewage, mostly in China, with only two found in urinary tract *E. coli* strains from Pennsylvania (USA) (Table S1) (12, 18). Eleven of the genes possess an identical combination of IS*26*-ΔIS*1294*-*gshB*-ΔIS*Sba14*, suggesting the presence of a common origin of *gshB* which has disseminated across several plasmid families (Table S1).

Subtyping of IncX group plasmids. IncX group plasmids are highly diverse between subgroups, thus posing a great challenge in plasmid typing (3). IncX plasmids were initially categorized into IncX1 and IncX2 subgroups, based on restriction analysis and replicon probes (1). In 2005, Carattoli et al. developed a PCR-based replicon typing method (PBRT) for plasmid typing which included the IncX group, with primers targeting the replication region (gamma origin) of IncX2 plasmid R6K (19). As more IncX plasmids were fully sequenced, a revised PBRT was developed to classify subgroups IncX1 to IncX4 on the basis of *taxC* genes (3), and then the replication genes (*pir* and *bis*) were used for the typing of subgroups IncX1 to IncX8 in PlasmidFinder, a Web tool for *in silicon* detection and typing of plasmids (5). Recently, Chen et al. and Du et al. reported bla_{KPC}-bearing IncX5 and IncX6 plasmids (pBK31567, GenBank accession number JX193302.1; pKPC3_SZ, KU302800.1), classified by *taxC* genes and conserved backbone sequences, which are now included in PlasmidFinder as IncX5 and IncX6 plasmids on the basis of *pir* genes (Fig. S2) (4, 6). We therefore performed a phylogenetic analysis to compare the backbone genes *taxC*, *pir*, and *bis* of 33 IncX plasmids, including pYD786-4, pYD786-3, plasmid C, pMCR1_IncX4, pNDM5_IncX3, pCQ02-121, pESTMCR (IncX4; GenBank accession number KU743383.1), pHS696_34 (IncX4; JX258654.1), pJARS35 (IncX7; NC_015054.1), pHI4320 (IncX8; AM942760.1), and 23 IncX plasmids, as described by Du et al. (6). All of them were subtyped based on the criteria of PlasmidFinder, with four reference *bis* genes for the IncX1 subgroup and nine reference *pir* genes for the subgroups IncX2 to IncX8. The phylogeny of 32 *taxC* genes was almost consistent with that of *bis*, except that the IncX1-X2 hybrid plasmids (pYD786-3 and plasmid C) and the IncX4-X3 hybrid plasmid pCQ02-121 were grouped into IncX2 and IncX3 clusters, respectively, in the *taxC* tree (Fig. S2). pYD786-4 was not included in the *taxC* tree due to the loss of *taxC*. In the *pir* tree, IncX3 and IncX4 plasmids were grouped into two clusters which were consistent with the criteria of PlasmidFinder as two kinds of IncX3 and IncX4 plasmids were used as references, and IncX1 plasmids were grouped into four clusters based on the diversity of *pir* genes in this subgroup. Hybridization events between IncX1-X2 and IncX4-X3 plasmids have led to a separation of the DNA transfer gene locus (including *taxC*) from the replication region of the same subgroup (Fig. S2), resulting in confusion when subtyping is performed with different typing markers. Also, we found an IncX2 plasmid without the *pilX-tax* module, providing additional evidence that replication genes might be the preferred targets for discrimination of the IncX group plasmids as used in the PlasmidFinder Web tool (5).

MATERIALS AND METHODS

E. coli YD786 carries four plasmids including *fosA6*-harboring pYD786-2 (GenBank accession no. KU254579.1), $bla_{CTX\text{-}M\text{-}2}$-harboring pYD786-1 (KU254578.1), and two IncX plasmids (pYD786-3 and pYD786-4), which were sequenced and assembled as reported before (12, 20). BLAST tools (http://blast.ncbi.nlm.nih.gov/Blast.cgi) were used to search for homologues of IncX plasmid backbone. The reference genome of plasmid R6K is available via the Sanger Institute's website (http://www.sanger.ac.uk/resources/downloads/plasmids/). Easyfig, version 2.2, was used for comparison of related plasmids. Phylogenetic analysis was performed using MEGA, version 7 (21).

Accession number(s). The nucleotide sequences of pYD786-3 and pYD786-4 are available in the GenBank database under accession numbers KU254580.1 and KU254581.1, respectively.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.00536-17.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

ACKNOWLEDGMENTS

Q.G. was supported by grant numbers 81673479 and 81120108024 from the National Natural Science Foundation of China and grant number 16PJD010 from the Shanghai Pujiang Program. N.S. is funded by a University of Oxford Clinical Lectureship. Part of the analysis was supported by a research grant from the National Institutes of Health (R21AI123747).

The funders had no role in study design, analysis, or preparation of the manuscript.

Y.D. has served on advisory boards for Achaogen, Allergan, Curetis, The Medicines Company, and Roche and received research funding from The Medicines Company for studies unrelated to this work. No other authors have potential conflicts of interest.

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