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Genetic Characterization of IncI2 Plasmids Carrying $bla_{CTX\text{-}M\text{-}55}$ Spreading in both Pets and Food Animals in China

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pHN1122-1 carrying $bla_{CTX\text{-}M\text{-}55}$, from an *Escherichia coli* isolate from a dog, was completely sequenced. pHN1122-1 has an IncI2 replicon and typical IncI2-associated genetic modules, including *mok*/*hok*-*finO*-*yafA*/*B*, *nikABC*, and two transfer regions, *tra* and *pil*, as well as a shufflon. $bla_{CTX\text{-}M\text{-}55}$ is found within a 3.084-kb IS*Ecp1* transposition unit that includes a fragment of IncA/C plasmid backbone. pHN1122-1 and closely related plasmids were identified in other *E. coli* isolates from animals in China.

The dissemination of CTX-M enzymes conferring resistance to extended-spectrum β-lactam antibiotics around the world has been referred to as the "CTX-M pandemic" (1). Within this group, the most dominant variants are $bla_{CTX\text{-}M\text{-}15}$ and $bla_{CTX\text{-}M\text{-}14}$, found in isolates from humans, animals, and the environment all over the world (2, 3). In China, CTX-M-14 is the dominant extended-spectrum β-lactamase (ESBL) among human and animal isolates (4–6), but CTX-M-55, rather than CTX-M-15, has become the second most frequently identified CTX-M gene, especially in *Escherichia coli* from animals (5–8). Like $bla_{CTX\text{-}M\text{-}15}$, $bla_{CTX\text{-}M\text{-}55}$ encodes an ESBL with enhanced activity against ceftazidime and is generally found in an IS*Ecp1*-mediated transposition unit carried by plasmids (9). However, no such plasmid has been fully sequenced. Here, a representative plasmid carrying $bla_{CTX\text{-}M\text{-}55}$, isolated from a dog (5) and designated pHN1122-1, was fully sequenced and other plasmids carrying $bla_{CTX\text{-}M\text{-}55}$ were compared with pHN1122-1 by PCR and restriction digestion.

Previously, 24 of 240 *E. coli* isolates from pets were found to produce CTX-M-55 and 15 gave transconjugants carrying $bla_{CTX\text{-}M\text{-}55}$ that were all resistant to cefotaxime and ceftazidime (5). None of these 15 isolates produced amplicons by PCR-based replicon typing (PBRT) using the 18 primer pairs described previously (10). Plasmid DNA from a transconjugant of *E. coli* 122 recovered from a dog in Guangzhou in 2007 (Table 1) (5) was extracted using a Qiagen Midi kit (Qiagen, Hilden, Germany) and sequenced on an ABI 3730xl sequencer (Applied Biosystems). Assembly with the Phred/Phrap/Consed software suite (University of Washington, Seattle, WA) gave four contigs. Combinatorial PCR to assemble contigs and fill in gaps gave a complete 62.196-kb plasmid.

BLASTn searches revealed that the gross structure of pHN1122-1 is closely related to that of the IncI2 plasmids R721 (GenBank accession no. AP002527) from *E. coli*, pSH146_65 from *Salmonella* Heidelberg (JN983044) (11), pChi7122-3 from *E. coli* APEC strain 7122 (O78:K80:H9) (FR851304) (12), and a plasmid from *E. coli* O157:H7 strain FRIK2000 (NZ_ACXO01000104, referred to as pFRIK2000 here) (13) (Fig. 1).

The full sequence of R721, which is not yet published, was used as a reference for the annotation of pHN1122-1 (see Table S1 in the supplemental material). The RepA replicon protein of pHN1122-1 shows >98% amino acid identity with RepA of R721, pChi7122-3, and pFRIK2000 and has only two nucleic acid differences from *repA* of pSH146_65. Like R721, pHN1122-1 carries genes encoding plasmid stability functions, including *mok*/*hok* and *yafA*/*yafB* (*parA*). The *mok*/*hok* genes have only three nucleotide differences from those of pSH146_65 and R721.

Like IncI1 plasmids (14), IncI2-type plasmids produce two types of pili. The thick pilus encoded by the *tra* operon is the primary pilus for conjugation transfer. The thin pilus encoded by the *pil* locus is similar to other type IV secretion systems and contributes to increased conjugation rates in liquid medium (15, 16). Both IncI1- and IncI2-type plasmids also contain a shufflon region that undergoes complex rearrangements mediated by Rci, a plasmid-encoded site-specific recombinase (17, 18). Like the four plasmids listed above, pHN1122-1 has the *tra* and *pil* loci and a shufflon (Fig. 1). Phylogenetic analysis using entire sequences of the five IncI2 plasmids after the removal of any mobile elements or concatenated sequences of genes shared by all five IncI2 plasmids (see Table S1 in the supplemental material) indicates that they fall into three lineages (Fig. 1; see Fig. S1 in the supplemental material). One contains pHN1122-1 and pFRIK2000, another contains pSH146_65 only, and a third contains pChi7122-3 and R721. Comparison with proteins from the typical IncI1 plasmid R64 showed that only a few have some similarity to those of IncI2 plasmids (see Table S1).

$bla_{CTX\text{-}M\text{-}55}$, the only known antibiotic resistance gene carried by pHN1122-1, is located in a 3.084-kb IS*Ecp1* transposition unit. This consists of a fragment related to the typical IS*Ecp1*-$bla_{CTX\text{-}M\text{-}15}$ transposition unit, except with a 45-bp spacer instead of the usual 48 bp and a 112-bp fragment matching IncA/C plasmid backbones beyond *orf477*Δ. This whole structure is inserted near *yajA*

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TABLE 1 Characteristics of isolates and plasmids carrying $bla_{CTX-M-55}$[a]

Isolate	Origin	Date of isolation	Location[b]	Spacer size (bp)[c]	Plasmid features		Reference
					Replicon type	ApaLI	
122	Dog feces	2007.11	GDPH1	45	IncI2	A1	5
203	Dog feces	2007.12	GDPH1	45	IncI2	A1	5
107	Dog feces	2008.1.18	GDPH3	45	IncI2	A1	5
129	Dog feces	2008.1.19	GDPH2	45	IncI2	A3	5
124	Dog feces	2008.1.19	GDPH2	45	IncI2	A3	5
143	Dog feces	2008.1.22	GDPH2	45	IncI2	A3	5
160	Dog feces	2008.1.24	GDPH3	45	IncI2	A1	5
212	Dog feces	2008.2.22	GDPH2	45	IncI2	A2	5
02R	Dog feces	2008.2.21	GDPH2	45	IncI2	A5	5
304	Dog feces	2008.3.28	GDPH1	45	IncI2	A1	5
312	Dog feces	2008.3.31	GDPH1	45	IncI2	A1	5
408	Dog skin	2008.4.12	GDPH1	45	IncI2	A2	5
066	Cat feces	2008.5.6	GDPH1	45	IncI2	A3	5
069	Dog feces	2008.5.6	GDPH1	45	IncI2	A5	5
096	Cat feces	2008.5.9	GDPH1	45	IncI2	A3	5
D71	Dog feces	2007.4.5	GDPH1	45	IncI2	A5	20
HD96	Duck feces	2006.12	GD	45	IncI2	A3	7
HD101	Duck feces	2006.12	GD	45	IncI2	A4	7
FW122	Duck feces	2006.12	GD	45	IncI2	A3	7
HD111	Duck feces	2006.12	GD	45	IncI2	A3	7
RD175	Duck feces	2006.12	GD	45	IncI2	A4	7
RD197	Duck feces	2006.12	GD	45	IncI2	A3	7
RD212	Duck feces	2006.12	GD	45	IncI2	A3	7
GS11	Chicken feces	2009.5	GS	45	IncI2	A1	6
JX138	Pig feces	2009.8	JX	45	IncI2	A5	6
SD06	Chicken liver	2009.5	SD	45	IncI2	A1	6
D40	Dog	2006.12.4	GDPH1	48	IncI1	B1	20
RD174	Duck feces	2006.12	GD	48	IncI1	B1	7
HLJ9	Calf feces	2009.6	HLJ	48	IncI1	B1	6
SD15	Chicken liver	2009.5	SD	48	IncI1	B2	6
HN10	Chicken feces	2009.7	HN	48	IncI1	C	6
ZQ06	Pigeon	2009.7	GD	48	F2:A−:B−	D	6
HN21	Pigeon feces	2009.7	HN	48	F2:A−:B−	D	6
SD13	Chicken liver	2009.5	SD	127	F33:A−:B−	E	6
ZCG09	Pigeon	2009.7	GD	127	IncN	F	6

[a] All of the isolates listed gave transconjugants carrying $bla_{CTX-M-55}$. For isolates transferring multiple plasmids (underlined), single plasmids for digestion were obtained by transformation. All transconjugants/transformants carrying $bla_{CTX-M-55}$ and a single plasmid from all isolates were resistant to cefotaxime and ceftazidime and were susceptible to gentamicin, amikacin, tetracycline, chloramphenicol, ciprofloxacin, and trimethoprim-sulfamethoxazole by agar dilution according to EUCAST (http://www.eucast.org/clinical_breakpoints).

[b] Different provinces are indicated as follows: GD, Guangdong; GS, Gansu; JX, Jiangxi; SD, Shangdong; HN, Hunan; HLJ, Heilongjiang. GDPH1 to GDPH3 indicate different pet hospitals.

[c] Length of the spacer region between the left end of IS*Ecp1* and the $bla_{CTX-M-55}$ start codon.

and is flanked by 5-bp direct repeats (GAAAA) characteristic of IS*Ecp1* transposition (Fig. 1). This configuration could be explained by the insertion of an IS*Ecp1*-$bla_{CTX-M-55}$ transposition unit into an IncA/C backbone and capture of the adjacent fragment in a subsequent transposition event (19).

R721 carries three resistance genes, *dfrA1*, *sat2*, and *aadA1*, in a class 2 integron associated with Tn*7*, while pSH146_65 carries bla_{CMY-2} also associated with IS*Ecp1*. The sites of these insertions in these three plasmids are different (Fig. 1), and pChi7122-3 and pFRIK2000 do not carry any known antibiotic resistance genes.

The remaining 14 transconjugants carrying $bla_{CTX-M-55}$ and 20 additional *E. coli* isolates carrying $bla_{CTX-M-55}$ from pets ($n = 2$) and food animals ($n = 18$; Table 1) from our other previous studies (6, 7, 20) were also examined for pHN1122-1-like plasmids. All 20 of the additional isolates gave transconjugants carrying $bla_{CTX-M-55}$ in streptomycin-resistant *E. coli* C600 with selection on cefotaxime (2 μg/ml) and streptomycin (2,000 μg/ml). Examination of alkaline lysis extracts of plasmid DNA from the 34 transconjugants on agarose gels revealed that some had multiple plasmids. For these isolates (Table 1), transformation into *E. coli* DH5α with selection on 2 μg/ml cefotaxime was used to obtain a single plasmid carrying $bla_{CTX-M-55}$, as verified by PCR and gel analysis.

Plasmids from the 24 transconjugants or transformants carrying $bla_{CTX-M-55}$ were analyzed for replicon type (10, 21). Primers designed to amplify conserved regions of the IncI2 backbone (*repA*, *rci*, *pilO*, *nikB*, and *finO*; Table 2) revealed that all 14 transconjugants/transformants from the original set of isolates and 11 from the additional 20 had IncI2 plasmids. Sequencing revealed that the *repA* genes of all 25 were 100% identical to *repA* of pHN1122-1. Further PCR and sequencing identified the same

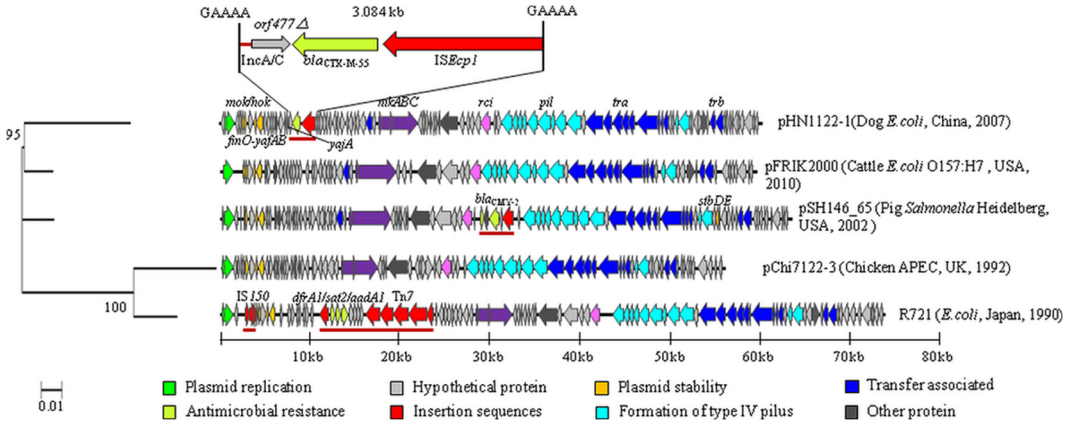

FIG 1 Linear comparisons of pHN1122-1 with other sequenced IncI2 plasmids. Mobile elements and/or resistance regions are underlined in red. The phylogeny was constructed in MEGA5 by the neighbor-joining method from the entire sequences of the five IncI2 plasmids after the removal of any mobile elements. Bootstrap consensus trees were inferred by using 500 replicates (values are shown next to nodes). Sequences with the following GenBank accession numbers were obtained: R721, AP002527; pSH146_65, JN983044; pChi7122-3, FR851304; pFRIK2000, NZ_ACXO01000104; pHN1122-1, JN797501. The maps were drawn by using Vector NTI-11.5.

IS*Ecp1*–$bla_{CTX\text{-}M\text{-}55}$ transposition unit with a 45-bp spacer in the same location as in pHN1122-1 in all 25. Digestion of plasmid DNA extracted from transconjugants or transformants with ApaLI (TaKaRa Biotechnology, China) revealed five slightly different plasmid types among the IncI2 plasmids (Table 1). These minor variations in ApaLI patterns may be due to mutations or recombination. IS*Ecp1*-$bla_{CTX\text{-}M\text{-}55}$ structures with a 48-bp or 127-bp spacer in the other nine transconjugants/transformants were associated with IncF, IncI1, or IncN plasmids (Table 1), suggesting that $bla_{CTX\text{-}M\text{-}55}$ genes with different spacer regions have been disseminated by different plasmid scaffolds.

The detection of pHN1122-1-like plasmids in isolates from companion and food animals from different geographic areas of China suggests that these plasmids are effective vectors for the dissemination of $bla_{CTX\text{-}M\text{-}55}$ among bacteria in different animal host species. As pHN1122-1 contains no obvious virulence or other antibiotic resistance genes, its persistence and spread may be attributed to either constant β-lactam exposure or its stability in the absence of any antimicrobial selection pressure. The addiction systems (*mok*/*hok*) and partitioning genes (*yafA*/*yafB*) that promote plasmid maintenance during vertical transmission, as well as the type IV pilus and shufflon, which have been shown to play a role in plasmid conjugation, epithelial cell adherence, and adherence to abiotic surfaces (17, 22), might contribute to the successful dissemination of these pHN1122-like plasmids.

Unlike IncI1 plasmids, IncI2 plasmids have not yet been well studied (11, 12), but this work suggests that they play an important role in the spread of $bla_{CTX\text{-}M\text{-}55}$. IncI2 plasmids carrying $bla_{CMY\text{-}2}$ (pSH146_65) and other resistance genes (R721) have also been identified, suggesting that IncI2 plasmids should be included in the group of plasmids involved in the dissemination of important drug resistance genes (23). Considering that IncI2 plasmids are widespread among different animal species but are probably currently missed in PBRT surveys, we suggest the inclusion of PCR screening for IncI2 plasmids to identify any associations with other important resistance genes. Further studies should also examine the prevalence and spread of these plasmids in the environment and human clinical settings.

Nucleotide sequence accession number. The nucleotide sequence of pHN1122-1 reported in this study has been deposited in the GenBank nucleotide sequence database under accession no. JN797501.

TABLE 2 Primers used to detect pHN1122-1-like regions

Primer	Nucleotide sequence (5′→3′)	Target DNA sequence	Position in pHN1122-1
RepA-F	CTGTCGGCATGTCTGTCTC	*repA* gene	923–941
RepA-R	CTGGCTACCAGTTGCTCTAA	*repA* gene	1475–1456
CHP1-F	GCTAAATGCTTCGCAGGAG	Upstream of *orf477*Δ	7093–7111
Orf477-R	ATTCAGCACCACGAAACGA	*orf477*Δ gene	8034–8052
ISEcp-F	TATTGTAGCATCGGTTTCC	*tnpA* of IS*Ecp1*	10274–10292
HP2-R	TGTTGTCCCGTATCCTTAT	Downstream of IS*Ecp1*	11595–11577
finO-F	CCCGTGATTGTGGTCAAA	*finO* gene	3280–3297
finO-R	GGGTTATCCGCCAGGTAT	*finO* gene	3597–3614
nikB-F	ATCCAACCTACAGACGCCTTAC	*nikB* gene	20234–20255
nikB-R	CTGCGACCTGTGCTTGCT	*nikB* gene	21151–21169
rci-F	TGCCCGTTTCTGTTCTCG	*rci* gene	30214–30231
rci-R	TGCCCTGTTGTCATCATTATTC	*rci* gene	30867–30888
pilQ-F	CGTTGGCGTGTAAGGTCG	*pilQ* gene	36773–36790
pilQ-R	CCTGGCGAAAGCAAACAA	*pilQ* gene	37513–37530

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REFERENCES

1. **Cantón R, Coque TM.** 2006. The CTX-M β-lactamase pandemic. Curr. Opin. Microbiol. **9**:466–475.
2. **Bush K, Fisher JF.** 2011. Epidemiological expansion, structural studies, and clinical challenges of new β-lactamases from Gram-negative bacteria. Annu. Rev. Microbiol. **65**:455–478.
3. **Cantón R, Gonzalez-Alba JM, Galan JC.** 2012. CTX-M enzymes: origin and diffusion. Front. Microbiol. **3**:110. doi:10.3389/fmicb.2012.00110.
4. **Hawkey PM, Jones AM.** 2009. The changing epidemiology of resistance. J. Antimicrob. Chemother. **64**(Suppl. 1):i3–i10.
5. **Sun Y, Zeng Z, Chen S, Ma J, He L, Liu Y, Deng Y, Lei T, Zhao J, Liu JH.** 2010. High prevalence of bla_{CTX-M} extended-spectrum β-lactamase genes in *Escherichia coli* isolates from pets and emergence of CTX-M-64 in China. Clin. Microbiol. Infect. **16**:1475–1481.
6. **Zheng H, Zeng Z, Chen S, Liu Y, Yao Q, Deng Y, Chen X, Lv L, Zhuo C, Chen Z, Liu JH.** 2012. Prevalence and characterisation of CTX-M β-lactamases amongst *Escherichia coli* isolates from healthy food animals in China. Int. J. Antimicrob. Agents **39**:305–310.
7. **Ma J, Liu JH, Lv L, Zong Z, Sun Y, Zheng H, Chen Z, Zeng ZL.** 2012. Characterization of extended-spectrum β-lactamase genes found among *Escherichia coli* isolates from duck and environmental samples obtained on a duck farm. Appl. Environ. Microbiol. **78**:3668–3673.
8. **Yu F, Chen Q, Yu X, Li Q, Ding B, Yang L, Chen C, Qin Z, Parsons C, Zhang X, Huang J, Luo Y, Wang L, Pan J.** 2011. High prevalence of extended-spectrum β lactamases among *Salmonella enterica* Typhimurium isolates from pediatric patients with diarrhea in China. PLoS One **6**:e16801. doi:10.1371/journal.pone.0016801.
9. **Zhao WH, Hu ZQ.** 2013. Epidemiology and genetics of CTX-M extended-spectrum β-lactamases in Gram-negative bacteria. Crit. Rev. Microbiol. **39**:79–101.
10. **Carattoli A, Bertini A, Villa L, Falbo V, Hopkins K, Threlfall EJ.** 2005. Identification of plasmids by PCR-based replicon typing. J. Microbiol. Methods **63**:219–228.
11. **Han J, Lynne AM, David DE, Tang H, Xu J, Nayak R, Kaldhone P, Logue CM, Foley SL.** 2012. DNA sequence analysis of plasmids from multidrug resistant *Salmonella enterica* serotype Heidelberg isolates. PLoS One **7**:e51160. doi:10.1371/journal.pone.0051160.
12. **Mellata M, Maddux JT, Nam T, Thomson N, Hauser H, Stevens MP, Mukhopadhyay S, Sarker S, Crabbe A, Nickerson CA, Santander J, Curtiss R, III.** 2012. New insights into the bacterial fitness-associated mechanisms revealed by the characterization of large plasmids of an avian pathogenic *E. coli*. PLoS One **7**:e29481. doi:10.1371/journal.pone.0029481.
13. **Dowd SE, Crippen TL, Sun Y, Gontcharova V, Youn E, Muthaiyan A, Wolcott RD, Callaway TR, Ricke SC.** 2010. Microarray analysis and draft genomes of two *Escherichia coli* O157:H7 lineage II cattle isolates FRIK966 and FRIK2000 investigating lack of Shiga toxin expression. Foodborne Pathog. Dis. **7**:763–773.
14. **Johnson TJ, Shepard SM, Rivet B, Danzeisen JL, Carattoli A.** 2011. Comparative genomics and phylogeny of the IncI1 plasmids: a common plasmid type among porcine enterotoxigenic *Escherichia coli*. Plasmid **66**:144–151.
15. **Kim SR, Komano T.** 1997. The plasmid R64 thin pilus identified as a type IV pilus. J. Bacteriol. **179**:3594–3603.
16. **Bradley DE.** 1984. Characteristics and function of thick and thin conjugative pili determined by transfer-derepressed plasmids of incompatibility groups I1, I2, I5, B, K and Z. J. Gen. Microbiol. **130**:1489–1502.
17. **Garcillán-Barcia MP, Francia MV, de la Cruz F.** 2009. The diversity of conjugative relaxases and its application in plasmid classification. FEMS Microbiol. Rev. **33**:657–687.
18. **Kim SR, Komano T.** 1992. Nucleotide sequence of the R721 shufflon. J. Bacteriol. **174**:7053–7058.
19. **Partridge SR.** 2011. Analysis of antibiotic resistance regions in Gram-negative bacteria. FEMS Microbiol. Rev. **35**:820–855.
20. **Deng Y, He L, Chen S, Zheng H, Zeng Z, Liu Y, Sun Y, Ma J, Chen Z, Liu JH.** 2011. F33:A−:B− and F2:A−:B− plasmids mediate dissemination of *rmtB*-$bla_{CTX-M-9}$ group genes and *rmtB-qepA* in *Enterobacteriaceae* isolates from pets in China. Antimicrob. Agents Chemother. **55**:4926–4929.
21. **Villa L, García-Fernández A, Fortini D, Carattoli A.** 2010. Replicon sequence typing of IncF plasmids carrying virulence and resistance determinants. J. Antimicrob. Chemother. **65**:2518–2529.
22. **Dudley EG, Abe C, Ghigo JM, Latour-Lambert P, Hormazabal JC, Nataro JP.** 2006. An IncI1 plasmid contributes to the adherence of the atypical enteroaggregative *Escherichia coli* strain C1096 to cultured cells and abiotic surfaces. Infect. Immun. **74**:2102–2114.
23. **Carattoli A.** 2009. Resistance plasmid families in *Enterobacteriaceae*. Antimicrob. Agents Chemother. **53**:2227–2238.