AMERICAN SOCIETY FOR MICROBIOLOGY | Antimicrobial Agents and Chemotherapy®

Polymorphism Existence of Mobile Tigecycline Resistance Gene *tet*(X4) in *Escherichia coli*

Huangwei Song,[a] Dejun Liu,[a] Ruichao Li,[b] Yulin Fu,[a] Weishuai Zhai,[a] Xiao Liu,[a] Tao He,[c] Congming Wu,[a] Li Bai,[d] Yang Wang[a]

[a]Beijing Advanced Innovation Center for Food Nutrition and Human Health, College of Veterinary Medicine, China Agricultural University, Beijing, China
[b]Jiangsu Co-Innovation Center for Prevention and Control of Important Animal Infectious Diseases and Zoonoses, College of Veterinary Medicine, Yangzhou University, Yangzhou, China
[c]Key Laboratory for Control Technology and Standard for Agro-product Safety and Quality, Ministry of Agriculture and Rural Affairs, Jiangsu Key Laboratory for Food Quality and Safety—State Key Laboratory Cultivation Base of Ministry of Science and Technology, Institute of Food Safety and Nutrition, Jiangsu Academy of Agricultural Sciences, Nanjing, China
[d]Key Laboratory of Food Safety Risk Assessment, National Health Commission of the People's Republic of China, China National Center for Food Safety Risk Assessment, Beijing, China

Huangwei Song and Dejun Liu contributed equally to this work. Author order was determined by drawing straws.

KEYWORDS tigecycline resistance, *tet*(X4) gene, MinION sequencing

Recently, two novel plasmid-mediated tigecycline resistance genes, *tet*(X3) and *tet*(X4), were identified in *Acinetobacter baumannii* and *Escherichia coli*, respectively, both of which can significantly compromise the efficacy of tigecycline (1–3), which is one of the few available drugs that can be used to treat infections caused by extensively drug-resistant pathogens (4). The presence of *tet*(X) variant genes on conjugative plasmids significantly increases the speed of dissemination of tigecycline resistance among these pathogens (1). Furthermore, several studies indicated that IS*CR2* may mediate the transposition process of *tet*(X4) (5, 6). Despite these initial reports, the existence of *tet*(X) genes and the mechanisms of their transfer need further exploration.

During our annual surveillance of antibiotic resistance among bacteria of food-producing animal origin in Guangdong Province, China, in 2018, a *tet*(X4)-positive strain 16EC was identified. The strain was recovered from a fecal swab sample from an apparently healthy pig and was identified as *E. coli* using both matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonik GmbH, Bremen, Germany) and 16S rRNA gene sequencing. Antimicrobial susceptibility testing was performed using the broth microdilution method according to the Clinical and Laboratory Standards Institute document M100S-S25 (7, 8) and with the EUCAST breakpoint (http://www.eucast.org/clinical_breakpoints/) for tigecycline. Strain 16EC was resistant to all tested tetracycline antibiotics, including tigecycline (MIC = 32 mg/liter), but susceptible to colistin, meropenem, ciprofloxacin, ceftazidime, and cefepime (see Table S1 in the supplemental material). S1-pulsed-field gel electrophoresis (S1-PFGE) and Southern blotting were performed to determine the location of *tet*(X4) in the genome of 16EC, the result of which located *tet*(X4) on the bands with sizes of ~125 kb, ~30 kb, and ~9 kb (Fig. S1).

The genomic DNA of 16EC was then subjected to 300-bp paired-end whole-genome sequencing using the Illumina HiSeq 2500 system (Annoroad, Beijing, China), while the plasmid was sequenced using the MinION system (Oxford Nanopore Technologies, Oxford, UK); then, the high-quality *de novo* assembly was performed with Unicycler v0.4.7, which simultaneously combines short-read (Illumina reads) and long-read (MinION reads) data (9–11). A total of 0.5 Gb and 1.08 Gb of clean data were obtained from the Illumina sequencing and MinION sequencing

Citation Song H, Liu D, Li R, Fu Y, Zhai W, Liu X, He T, Wu C, Bai L, Wang Y. 2020. Polymorphism existence of mobile tigecycline resistance gene *tet*(X4) in *Escherichia coli*. Antimicrob Agents Chemother 64:e01825-19. https://doi.org/10.1128/AAC.01825-19.

Address correspondence to Yang Wang, wangyang@cau.edu.cn.

Accepted manuscript posted online 2 December 2019
Published 27 January 2020

platforms, respectively. The N50 value of the MinION sequencing was 18,245 bp. Sequencing results revealed the presence of three plasmids in 16EC, the 126,656-bp p0111-type plasmid p16EC-p0111, the 124,991-bp IncN-type plasmid p16EC-IncN, and a smaller 9,228-bp plasmid, p16EC-9K. p16EC-9K contained one intact copy of IS*CR2* along with six other open reading frames (ORFs), encoding a Tet(X4), a replicon protein, a hydrolase, and three hypothetical proteins (Fig. 1). The *rep* gene showed 84% nucleotide sequence identity and 86% amino acid identity to the *rep* region of the ColE2-type plasmid pETEC58 (GenBank accession number FN649416.1) from *E. coli* (12). To further confirm the function of ColE2-like *rep* and to exclude the interference of IS*CR2* in the transfer of p16EC-9K, we ligated a partial sequence of p16EC-9K without the IS*CR2* element with an ampicillin resistance gene, *ampR*, and transformed the recombinant plasmid into DH5α. The *tet*(X4)-positive and IS*CR2*-negative transformants exhibited resistance to tigecycline (MIC, 16 mg/liter). S1-PFGE and Southern blotting results verified the existence of this recombinant plasmid in transformant (Fig. S2). These results confirmed the function of the ColE2-like replicon of p16EC-9K in *E. coli*.

Although sequencing data showed that *tet*(X4) was only located on the p16EC-9K plasmid, the ~125-kb *tet*(X4)-carrying band confirmed by Southern blotting was missing from the two assembled plasmids, p16EC-p0111 and p16EC-IncN, of 16EC. To probe the reason, original reads of ≥20 kb were subjected to BLASTn comparison against the *tet*(X4), with strict cutoffs for alignment length and identity (coverage, ≥90%; identity, ≥85%). A total of 111 *tet*(X4)-positive reads of >20 kb in size were selected following screening of 47,151 original MinION sequencing reads and compared against the sequences of the two 16EC plasmids, p16EC-p0111 and p16EC-IncN, using BLASTn analysis. Six (37 kb to 90 kb) and one (34 kb) of the 111 reads showed high similarity to regions within p16EC-p0111 and p16EC-IncN, respectively (Fig. S3). The *tet*(X4)-carrying fragments in these seven reads showed high nucleotide sequence identity (>85%) to that of p16EC-9K (coverage, >99%). However, the arrangement of the p16EC-9K-associated ORFs differed among the long reads; IS*CR2* was located upstream of *tet*(X4) in the p16EC-IncN reads but was located downstream of *tet*(X4) in the p16EC-p0111 reads (Fig. 1A). These results confirmed that *tet*(X4) was also located on the two ~125-kb plasmids (p16EC-p0111 and p16EC-IncN), which is consistent with the result of Southern blotting. However, it remains largely unclear why the direction of p16EC-9K insertion into the two plasmids differed and how these processes occurred. We propose two possible formation processes. (i) In plasmid fusion, p16EC-9K was fused into p16EC-p0111 and p16EC-IncN mediated by IS*1* and IS*1294*, respectively, through recombination (Fig. 1A). (ii) In transposition, p16EC-9K was transited into these two large plasmids by IS*CR2*, which differed from other insertion sequences in that it lacked terminal inverted repeats and is thought to transpose via a mechanism termed rolling-circle transposition (13, 14). As such, *tet*(X4) could theoretically be transposed using a single intact copy of IS*CR2*. Furthermore, the low number of *tet*(X4)-positive p16EC-p0111 ($n = 6$) and p16EC-IncN ($n = 1$) reads might account for why the *tet*(X4) gene was absent on these plasmids following *de novo* assembly of the MinION and Illumina HiSeq sequencing reads.

Unexpectedly, 104 of 111 original reads with various copy numbers of a tandem structure consisting of IS*CR2* and *tet*(X4) [IS*CR2*-*tet*(X4)] were observed (Fig. 1B). Considering the size and the composition of these repeats, it seems likely that they were formed by the fusion of multiple p16EC-9K plasmids, which may be generated by rolling-circle replication through IS*CR2*. The results of Southern blotting, as the ~30-kb *tet*(X4)-positive band, which may contain 3 copies of *tet*(X4)-IS*CR2*, further confirmed the existence of these structures. However, the specific mechanism remains unclear.

In conclusion, we characterized the polymorphism existence of the mobile tigecycline-resistant gene *tet*(X4) in *E. coli* 16EC. *tet*(X4) was located on three different Inc plasmids, p16EC-p0111, p16EC-IncN, and a novel ColE2-like plasmid, p16EC-9K, the

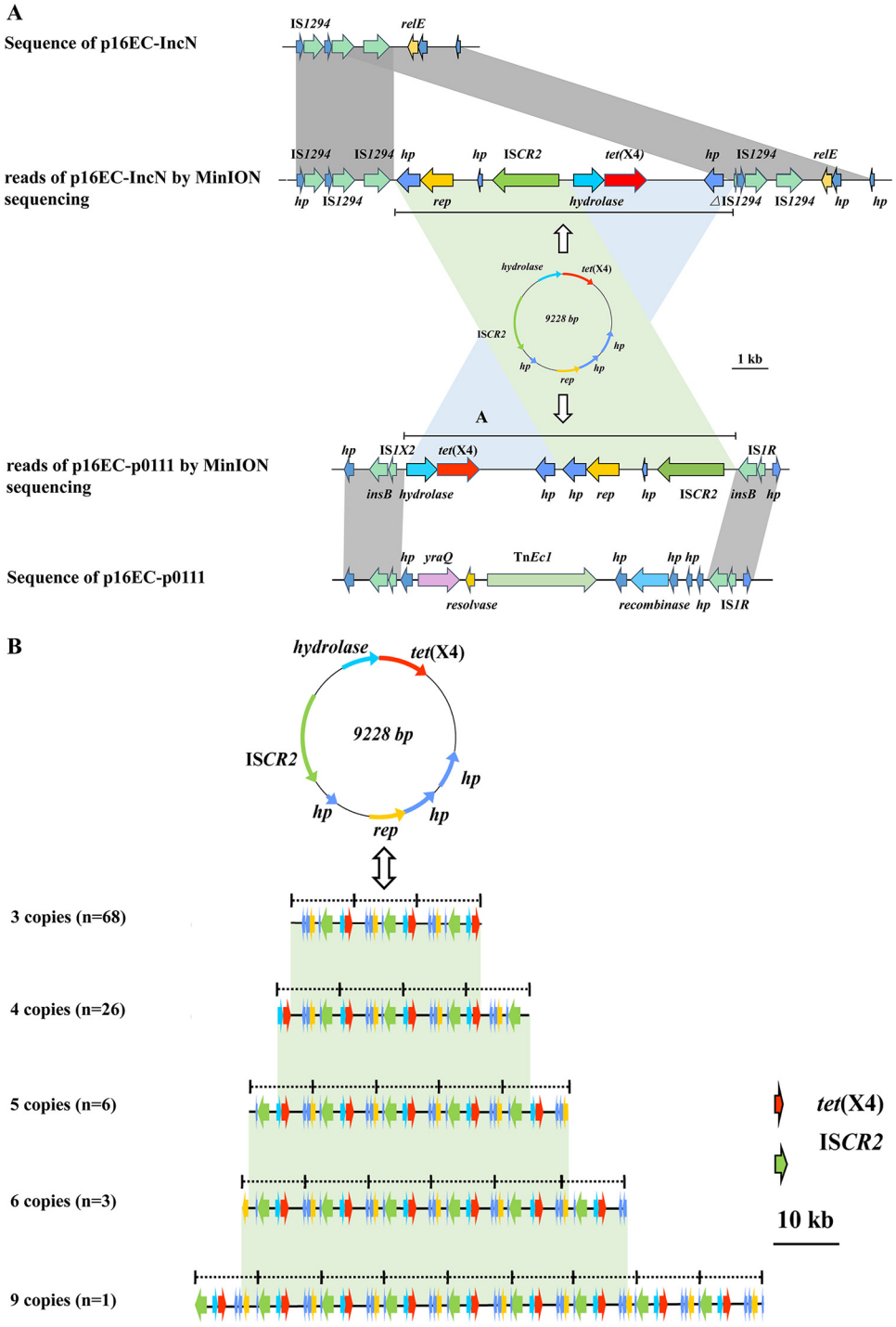

FIG 1 Characteristics of the novel ColE2-like plasmid p16EC-9K bearing *tet*(X4) and the tandem structure of multicopy of IS*CR2*-*tet*(X4). Arrows indicate genes and their directions. Regions in gray represent two linked areas with high sequence similarity. (A) Linear alignment of the original plasmid reads generated by MinION sequencing and the final polished sequences generated by HiSeq sequencing. A schematic of p16EC-9K is shown between the linear sequences. (B) Structure of the tandem *tet*(X4)-IS*CR2* segment and diagram showing the various copy number arrangements detected in the sequencing reads.

latter of which may play an important role in the mobilization of *tet*(X4) through either recombination or rolling-circle transposition via insertion sequence (IS) elements. Further studies are needed to fully understand the mechanism of multicopy variant formation and the transfer mode of the *tet*(X4) gene.

Data availability. The complete sequences of the plasmids p16EC-9K, p16EC-p0111, and p16EC-IncN have been deposited in NCBI under the GenBank accession numbers MN381965, MN086777, and MN086778, respectively. The original reads of p16EC-p0111 and p16EC-IncN containing *tet*(X4) have been deposited in the Figshare database (https://figshare.com/s/0b1391fe51e66212c58c).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

ACKNOWLEDGMENTS

This work was supported in part by the National Key R&D Program of China (2018YFD0500300) and the National Natural Science Foundation of China (81861138051 and 81661138002).

REFERENCES

1. He T, Wang R, Liu D, Walsh TR, Zhang R, Lv Y, Ke Y, Ji Q, Wei R, Liu Z, Shen Y, Wang G, Sun L, Lei L, Lv Z, Li Y, Pang M, Wang L, Sun Q, Fu Y, Song H, Hao Y, Shen Z, Wang S, Chen G, Wu C, Shen J, Wang Y. 2019. Emergence of plasmid-mediated high-level tigecycline resistance genes in animals and humans. Nat Microbiol 4:1450–1456. https://doi.org/10.1038/s41564-019-0445-2.
2. Sun J, Chen C, Cui CY, Zhang Y, Liu X, Cui ZH, Ma XY, Feng Y, Fang LX, Lian XL, Zhang RM, Tang YZ, Zhang KX, Liu HM, Zhuang ZH, Zhou SD, Lv JN, Du H, Huang B, Yu FY, Mathema B, Kreiswirth BN, Liao XP, Chen L, Liu YH. 2019. Plasmid-encoded tet(X) genes that confer high-level tigecycline resistance in Escherichia coli. Nat Microbiol 4:1457–1464. https://doi.org/10.1038/s41564-019-0496-4.
3. Bai L, Du P, Du Y, Sun H, Zhang P, Wan Y, Lin Q, Fanning S, Cui S, Wu Y. 2019. Detection of plasmid-mediated tigecycline-resistant gene tet(X4) in Escherichia coli from pork, Sichuan and Shandong Provinces, China, February 2019. Eurosurveillance 24:1900340. https://doi.org/10.2807/1560-7917.ES.2019.24.25.1900340.
4. Brust K, Evans A, Plemmons R. 2014. Favourable outcome in the treatment of carbapenem-resistant Enterobacteriaceae urinary tract infection with high-dose tigecycline. J Antimicrob Chemother 69:2875–2876. https://doi.org/10.1093/jac/dku185.
5. Chen C, Chen L, Zhang Y, Cui CY, Wu XT, He Q, Liao XP, Liu YH, Sun J. 2019. Detection of chromosome-mediated tet(X4)-carrying Aeromonas caviae in a sewage sample from a chicken farm. J Antimicrob Chemother 74:3628–3630. https://doi.org/10.1093/jac/dkz387.
6. Chen C, Cui CY, Zhang Y, He Q, Wu XT, Li G, Liao XP, Kreiswirth BN, Liu YH, Chen L, Sun J. 2019. Emergence of mobile tigecycline resistance mechanism in Escherichia coli strains from migratory birds in China. Emerg Microbes Infect 8:1219–1222. https://doi.org/10.1080/22221751.2019.1653795.
7. CLSI. 2018. Performance standards for antimicrobial susceptibility testing; 25th informational supplement M100-S25. Clinical and Laboratory Standards Institute, Wayne, PA.
8. CLSI. 2013. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals; approved standard, 4th ed, VET01-A4 and supplement VET01-S2. Clinical and Laboratory Standards Institute, Wayne, PA.
9. Wick RR, Judd LM, Gorrie CL, Holt KE. 2017. Unicycler: resolving bacterial genome assemblies from short and long sequencing reads. PLoS Comput Biol 13:e1005595. https://doi.org/10.1371/journal.pcbi.1005595.
10. Li R, Xie M, Dong N, Lin D, Yang X, Wong MHY, Chan EW, Chen S. 2018. Efficient generation of complete sequences of MDR-encoding plasmids by rapid assembly of MinION barcoding sequencing data. Gigascience 7:1–9. https://doi.org/10.1093/gigascience/gix132.
11. Li R, Chen K, Chan EW, Chen S. 2019. Characterization of the stability and dynamics of Tn6330 in an Escherichia coli strain by nanopore long reads. J Antimicrob Chemother 74:1807–1811. https://doi.org/10.1093/jac/dkz117.
12. Crossman LC, Chaudhuri RR, Beatson SA, Wells TJ, Desvaux M, Cunningham AF, Petty NK, Mahon V, Brinkley C, Hobman JL, Savarino SJ, Turner SM, Pallen MJ, Penn CW, Parkhill J, Turner AK, Johnson TJ, Thomson NR, Smith SG, Henderson IR. 2010. A commensal gone bad: complete genome sequence of the prototypical enterotoxigenic Escherichia coli strain H10407. J Bacteriol 192:5822–5831. https://doi.org/10.1128/JB.00710-10.
13. Jacques M, Michael C. 2005. Insertion sequences. Microbiol Mol Biol R 62:725–774.
14. Toleman MA, Bennett PM, Walsh TR. 2006. ISCR elements: novel gene-capturing systems of the 21st century? Microbiol Mol Biol Rev 70:296–316. https://doi.org/10.1128/MMBR.00048-05.