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Plasmid-encoded *tet*(X) genes that confer high-level tigecycline resistance in *Escherichia coli*

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Abstract

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Author contributions

JS and CC contributed equally in this study. Y-HL, LC, X-PL, and JS designed the study. CC, C-YC, YZ, XL, Z-HC, X-YM, K-XZ, H-ML, Z-HZ, S-DZ, J-NL, HD, BH, and F-YY collected the data. JS, CC, Y-JF, L-XF, X-LL, R-MZ, and Y-ZT analyzed and interpreted the data. Y-HL, BM, BNK, LC, JS, X-PL, and CC wrote the draft of the manuscript. All authors reviewed, revised, and approved the final report.

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Competing interests

The authors declare no competing interests.

Tigecycline is one of the last-resort antibiotics to treat complicated infections caused by both multidrug-resistant (MDR) Gram-negative and Gram-positive bacteria1. Tigecycline resistance has sporadically occurred in recent years, primarily due to chromosome-encoding mechanisms, such as overexpression of efflux pumps and ribosome protection[2,3]. Here we report the emergence of plasmid-mediated mobile tigecycline resistance mechanism Tet(X4) in *Escherichia coli* isolates from China, which is capable of degrading all tetracyclines, including tigecycline and the FDA newly approved eravacycline. The *tet*(X4)-harboring IncQ1 plasmid is highly transferable, and can be successfully mobilized and stabilized in recipient clinical and laboratory strains of Enterobacteriaceae bacteria. It is noteworthy that *tet*(X4)-positive *E. coli* strains, including isolates co-harboring *mcr-1*, have been widely detected in pigs, chickens, soil, and dust samples in China. *In vivo* murine models demonstrated that the presence of Tet(X4) led to tigecycline treatment failure. Consequently, the emergence of plasmid-mediated Tet(X4) challenges the clinical efficacy of the entire family of tetracycline antibiotics. Importantly, our study raises concern that the plasmid-mediated tigecycline resistance may further spread into a variety of ecological niches and into clinical high-risk pathogens. Collective efforts are in urgent need to preserve the potency of these essential antibiotics.

The emergence and spread of antimicrobial resistance in Enterobacteriaceae bacteria pose a serious threat to human and animal health. Of special concern is the emerging resistance to carbapenems, since these agents are often regarded as the last line of effective therapy for the treatment of infections caused by MDR Gram-negative bacteria[4]. Antibiotic treatment options for carbapenem-resistant Enterobacteriaceae (CRE) bacteria are becoming limited, and colistin and tigecycline have been regarded as the final armamentarium[5]. However, the recent discovery of a plasmid-mediated colistin resistance gene, *mcr-1*, threatens the clinical utility of colistin to treat these infections, leaving tigecycline as one of the last options[6].

Tigecycline resistance has inevitably emerged over the recent years, mostly identified among extensively drug- and carbapenem-resistant isolates[7,8]. Decreased susceptibility to tigecycline is commonly associated with overexpression of efflux pumps of the resistance-nodulation-cell division protein family, such as AcrAB-TolC, OqxAB, and AdeABC[9–11]. Meanwhile, mutations in *plsC*, *rpsJ*, *trm*, *tet*(A), and *tet*(M) have also been found to decrease tigecycline susceptibility[12–16]. In contrast, tetracycline destructases, such as Tet(X), represent a unique enzymatic tetracycline inactivation mechanism[17]. Tet(X), the flagship tetracycline-inactivating enzyme[18], has been confirmed for *in vitro* activity in degrading all tetracyclines including tigecycline[19]. Since first described in obligate anaerobes *Bacteroides fragilis*, *tet*(X) and its orthologous genes [namely *tet*(X1) and *tet*(X2)] have now been detected in clinical Enterobacteriaceae and *Acinetobacter baumannii* isolates, but their distribution, genetic structure, and clinical significance remain to be explored[20,21]. In this study, we described a plasmid-mediated mobile tigecycline resistance gene *tet*(X4), and explored the prevalence of *tet*(X4)-positive *E. coli* strains in humans, food-producing animals, and surrounding environment in China (Fig. 1).

Initially, a tigecycline-resistant *E. coli* strain LHM10–1 (8 mg/L; Supplementary Table 1) was isolated from the stool sample in an intensive pig farm (Jiangxi, China) in July 2017. The tigecycline resistance could be successfully transferred into the recipient *E. coli* C600 (8

mg/L; Supplementary Table 1), suggesting that the gene encoding tigecycline resistance might be plasmid-borne. WGS of *E. coli* LHM10–1 showed that it belonged to sequence type (ST) 515, and contained one 4.81-Mb chromosome and six plasmids with sizes of 108,366 bp (pLHM10–1-p1, IncF), 98,254 bp (pLHM10–1-p2, untypeable), 33,301 bp (pLHM10–1-p3, IncX4), 28,942 bp (pLHM10–1-p4, untypeable), 28,519 bp (pLHM10–1-p5, untypeable), and 12,783 bp (pLHM10–1-p6, IncQ1).

A *tet*(X)-like gene [designated as *tet*(X4)], with 1,158 bp in length, was found to be located on the IncQ1 plasmid pLHM10–1-p6. The *tet*(X4) gene encoded a 385 amino acid protein (Fig. 2a; Supplementary Fig. 1a), which showed 94.5% amino acid sequence identity to Tet(X), initially found in the obligate anaerobe *B. fragilis* (GenBank accession number M37699). Meanwhile, Tet(X4) displayed 63.9%, 95.1%, and 85.7% identities to the other named Tet(X) orthologs Tet(X1) (AJ311171), Tet(X2) (AJ311171), and Tet(X3) (AB097942), respectively (Supplementary Fig. 1a).

Gene cloning experiment showed that the *tet*(X4) construct, namely *E. coli* JM109+pBAD24-*tet*(X4), demonstrated a 64-fold increase in the minimum inhibitory concentration (MIC) of tigecycline when compared with the host *E. coli* JM109 strain carrying pBAD24 (0.25 mg/L; Supplementary Table 1). Similarly, MICs of the first- and second-generation tetracyclines were increased at least 32-fold. In addition, MIC of the FDA newly approved eravacycline, which was structurally similar to tigecycline with two modifications at C-7 and C-9, increased 512-fold (4 mg/L; Supplementary Table 1). In addition, the agar well diffusion assay showed that the addition of supernatant from *E. coli* JM109+pBAD24-*tet*(X4) resulted in the elimination of tetracyclines inhibition zone, while there were no significant changes between the negative and blank controls, suggestive of inactivation of tetracyclines by Tet(X4) (Fig. 2b). Furthermore, LC-MS/MS showed that the level of tetracycline remained (16.1 ± 1.2)% after incubation with *E. coli* JM109+pBAD24-*tet*(X4) for 16 hours [verse (80.2 ± 1.2)% in the negative control] (Fig. 2c; $P < 0.0001$), and eravacycline remained only (7.8 ± 0.5)% after incubation for the same time [verse (79.0 ± 3.8)% in the negative control] (Fig. 2d; $P < 0.0001$). Our above results demonstrated that Tet(X4) was able to confer resistance to the entire family of tetracyclines.

In *E. coli* LHM10–1, the *tet*(X4)-harboring plasmid pLHM10–1-p6 was 12,783 bp in size with an average G+C content of 55.4% and contained 15 predicted ORFs, of which *tet*(X4) was adjacent to two copies of IS*CR2* (one is truncated; Fig. 3a). Plasmid replicon analysis showed that pLHM10–1-p6 belonged to the broad host-range IncQ1 group, possessing the IncQ1 plasmid backbone genes for plasmid replication (*repA*, *repB*, and *repC*) and mobilization (*mobA*, *mobB*, and *mobC*). A comparison of several IncQ1 plasmids from the public National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/) demonstrated that they shared similar replication and mobilization regions, but diverse acquired regions (between *repC* and *mobC*) (Fig. 3b). The results suggested that pLHM10–1-p6 originated by acquisition of *tet*(X4) into an IncQ1 plasmid, likely associated with the translocation of IS*CR2*.

Interestingly, a *mcr-1*-carrying IncX4 plasmid pLHM10–1-p3 (33,301 bp) was identified in the same *E. coli* LHM10–1, which shared >99% sequence identity and 100% query coverage

to several other *mcr-1*-harboring plasmids (e.g., KX084392 and CP018773) from animal and human sources in China, USA, and some other countries. In addition, an extended-spectrum β-lactamase (ESBL) gene $bla_{\text{CTX-M-14}}$ was identified on the chromosome of *E. coli* LHM10–1.

Further conjugation assays showed that tigecycline resistance could be successfully transferred from *E. coli* LHM10–1 into laboratory strains *E. coli* C600, *S. Typhimurium* ATCC 14028, and *K. pneumoniae* ATCC 700603, at a frequency of $(1.2 \pm 0.5)\times10^{-2}$, $(1.2 \pm 0.3)\times10^{-4}$, and $(6.2 \pm 1.3)\times10^{-2}$ cells per recipient cell, respectively (Fig. 3c). Notably, pLHM10–1-p6 also exhibited good transferability into clinical CRE strains, including NDM-5-producing ST167 *E. coli* 1314 and KPC-2-producing ST11 *K. pneumoniae* 1332, at the frequency of $(2.2 \pm 0.5)\times10^{-4}$ and $(8.0 \pm 0.5)\times10^{-6}$, respectively (Fig. 3c). MICs of tetracyclines, including tigecycline and eravacycline, among these transconjugants were increased at least 8-fold (Supplementary Table 1). After 22 days (approximately 220 generations) of serial passage without antibiotic treatment, pLHM10–1-p6 was still stably maintained (75%−100% fraction of plasmid-containing lineages) in the parental *E. coli* LHM10–1 and transconjugants hosts, including the clinical CRE strains (Fig. 3d). Given the high transferability and stability of *tet*(X4) in clinical CRE isolates harboring bla_{KPC} or bla_{NDM}, further acquisition of carbapenem resistance genes by *tet*(X4)-harboring strains, or alternatively, the spread of *tet*(X4)-carrying plasmids into clinical MDR strains, had the potential to create truly pan-drug resistant strains, resulting in untreatable infections.

In this study, the *tet*(X4) gene was totally detected in 42 *E. coli* strains [sharing 100% nucleotide sequence identity of *tet*(X4) with each other] from 4,189 samples, consisting of 30 (1.3%) from 2,337 pigs, 8 (0.8%) from 1,061 chickens, 2 (0.8%) from 256 soil samples, and 2 (0.9%) from 232 dust samples (Supplementary Table 2). These *tet*(X4)-positive *E. coli* isolates were found in five provinces in Southern and Eastern China (Fig. 1), including Guangdong (n=12), Fujian (n=11), Jiangsu (n=8), Jiangxi (n=6), and Guangxi (n=5). However, the *tet*(X4)-positive *E. coli* strains weren't detected in others.

Although we have not identified *tet*(X4)-positive *E. coli* strains from human sources, a series of bacterial genomes carrying *tet*(X4)-like genes from human (e.g., CP023968), animal (e.g., CP002562), water (e.g., CP023049), and soil (e.g., LT906465) sources were identified in NCBI (Supplementary Fig. 1b). Furthermore, the sequence analysis also indicated that the *tet*(X4)-like genes had spread outside China and into other countries, including USA (e.g., NZ_JYQO01000042), UK (e.g., CP000685), and Thailand (e.g., NZ_JRQZ01000088) (Supplementary Fig. 1b).

The susceptibility testing demonstrated that the 42 *tet*(X4)-positive *E. coli* strains were all resistant to tigecycline, tetracycline, sulfamethoxazole/trimethoprim, and florfenicol, while 15 (35.7%), 12 (28.6%), 10 (23.8%), and 9 (21.4%) isolates showed resistance to ciprofloxacin, cefotaxime, gentamicin, and colistin, respectively (Fig. 4). In addition, all isolates exhibited an eravacycline MIC of 4–8 mg/L, significantly higher than the MIC_{90} (0.5 mg/L) for *E. coli* strains from the routine surveillance[22]. Notably, the 12 cefotaxime-resistant isolates were all found to carry ESBL genes, including $bla_{\text{CTX-M-14}}$ (n=7), $bla_{\text{CTX-M-65}}$ (n=4), and $bla_{\text{CTX-M-27}}$ (n=1). The nine colistin-resistant *E. coli* strains all

carried colistin resistance gene *mcr-1* and ESBL gene $bla_{\text{CTX-M}}$ ($bla_{\text{CTX-M-14}}$ or $bla_{\text{CTX-M-65}}$), belonging to two STs (ST515 [n=5] and ST1196 [n=4]).

MLST showed that the 42 *tet*(X4)-positive *E. coli* strains belonged to nine distinct STs, which were in general consistent with the pulsotypes (A-K) from the PFGE analysis (Fig. 4). Strains from the same ST or PFGE pulsotype usually shared similar antimicrobial resistance profiles. Two STs (ST8302 and ST8338) were first identified in the current study, with ST8302 the most predominant ST (n=16; 38.1%) overall. Notably, the same STs of strains (ST8302, ST515, ST761, and ST10) in this study were identified in different geological regions and sources, suggesting the possibility of clonal spread of the same *tet*(X4)-positive strains across different regions.

The detection of IncQ1 pLHM10–1-p6-like plasmid revealed that 57.1% (24/42) of the *tet*(X4)-positive *E. coli* isolates harbored the same pLHM10–1-p6-like plasmid (Fig. 4). These strains belonged to four distinct STs (ST8302 [n=16], ST515 [n=6], ST761 [n=1], and ST871 [n=1]), and were isolated from four different provinces, including Guangdong (n=7), Jiangsu (n=7), Jiangxi (n=6), and Fujian (n=4). Most of them were collected from pigs (n=14) and chickens (n=7), followed by dusts (n=2) and soils (n=1).

Notably, IncQ-type plasmids are a group of relatively small (approximately 5 to 15 kb), non-conjugative but mobilizable plasmids that can be transferred into a broad range of bacterial hosts in the presence of self-transmissible helper plasmids[23]. Our mating experiment results showed that pLHM10–1-p6 could be successfully transferred in the presence of *mcr-1*-carrying IncX4 plasmid pZ6–2 (transfer frequency, $(5.4\pm1.8)\times10^{-2}$), IncI2 plasmid pHLJ179 ((7.9±1.5)×10–5) and IncFII plasmid pH12–4 ($(4.3\pm1.0)\times10^{-5}$) (Supplementary Table 3), but the transfer was failed in the absence of helper plasmids (pLHM10–1-p6 alone). Co-transfer of the helper plasmids along with pLHM10–1-p6 into the recipient *E. coli* C600 wasn't observed under the selection by tigecycline. Our results indicated that a helper plasmid is needed for the mobilization of *tet*(X4)-harboring pLHM10–1-p6-like plasmids, and importantly, common *mcr-1*-bearing plasmids may serve as helper plasmids to further facilitate the spread of plasmid-mediated tigecycline resistance.

Moreover, WGS of another colistin-resistant ST1196 strain *E. coli* G3X16–2 showed that it consisted of one 4.78-Mb chromosome and two plasmids in the sizes of 265,575 bp (pG3X16–2-p1) and 138,950 bp (pG3X16–2-p2), respectively. Plasmid pG3X16–2-p1 belonged to IncHI2 group, and harbored *mcr-1* and $bla_{\text{CTX-M-65}}$. The *tet*(X4) was located on an IncF plasmid (F-:A18:B-), pG3X16–2-p2, co-harboring *aadA1, aadA2, aph(3")-Ib, aph(6)-Id,* $bla_{\text{TEM-1B}}$*, erm*(42)*, cmlA1, floR, sul3, tet*(A)*, tet*(M), and *dfrA12* (Supplementary Fig. 2), which suggested that the tigecycline resistance may be co-selected by other antimicrobial resistances. Intriguingly, pG3X16–2-p2 harbored four tandem repeated IS*CR2-catD-tet*(X4)-IS*CR2* gene cassettes on the same plasmid, suggesting the *tet*(X4)-harboring genetic elements are highly active and may further transfer to other plasmids or chromosome.

Results of *in vivo* experiment showed that the mice infected with plasmid-cured isogeneic strain *E. coli* STB20–1S [*tet*(X4)-negative] were highly susceptible to tigecycline treatment,

with more than two $\log_{10}$(CFU/g) reduction in *E. coli* counts, while tigecycline had no significant effect on animals infected by *E. coli* STB20–1 after 24 hours of treatment (Fig. 2e; $P < 0.0001$). The results indicated that the presence of *tet*(X4) may contribute to tigecycline treatment failure.

In this study, the *tet*(X4)-positive *E. coli* strains were identified in pigs, chickens, and their surrounding environmental samples from different geographical regions, indicating that Tet(X4)-encoding tigecycline resistance might have already spread in China. We suspected that the usage of "older" generations of tetracyclines had largely contributed to the emergence of newer mobile tigecycline resistance. In China, tigecycline is only approved for the treatment of clinical infections in human whereas its usage in food-producing animals is not authorized. However, the first- and second-generation tetracyclines were widely used in the treatment of infections in food-producing animals and for growth promotion at sub-therapeutic doses in China, likely providing a selective pressure for the emergence of tigecycline resistance[24]. As one of the countries with the largest amount of antibiotics usage in the world, 12,000 tons (7.4%) of tetracyclines were consumed by China yearly (data from in 2013), and most of them eventually entered into environment[25]. Similarly in Europe, although the European Union (EU) had phased out the use of antibiotics as growth promoters, the latest ECDC/EFSA/EMA joint report (2017) on the integrated analysis of antibiotic consumption and antimicrobial resistance in bacteria revealed that tetracyclines were still the most sold antibiotics in Europe, and a statistically significant positive association between tetracycline consumption and resistance in *E. coli* strains was observed in food-producing animals[26]. Of concern, the selective pressure imposed by the continuous application of tetracyclines in veterinary practice could serve to maintain and spread the *tet*(X4)-like genes into pathogenic microorganisms.

The mobile antimicrobial resistance mechanisms, including Tet(X4), MCR, and different families of carbapenemase (e.g., NDM), represent a plasmid-mediated antibiotic crisis. But the reality is that only limited antibiotics on the market or in the pipeline are effective against these highly resistant organisms. Continuous selection pressure from the environment, veterinary usage, and clinical practice, will likely accelerate the spread of *tet*(X4) and other resistance genes. The horizontal transfer ability of the resistance genes and plasmids, such as the *tet*(X4)-harboring broad-host range IncQ1 plasmid, can be a challenge for hospital infection control and public health surveillance. Our study underscores the need for a "one-health" strategy (i.e., considering the span of humans, animals, and their environment) for antimicrobial resistance, as infectious disease surveillance and containment across different sectors is paramount. To make this last-resort drug available, the prevalence of *tet*(X4) in both human and veterinary fields should be considered by surveillance programs, along with judicious administration of tetracyclines in animals and the environments.

Methods

Sample collection and bacterial strains

This study was approved by the institutional review board (IRB) of hospital A (Huizhou Municipal Central Hospital) and South China Agricultural University (SCAU). The clinical

isolates from hospitals B, C, and D were retrospectively collected, and patient data were not included in this study, therefore ethical approval wasn't sought and obtained. The fecal samples were randomly collected from pigs (3 to 8 months old) and chickens (7 to 20 weeks old), with approximately 50 samples per farm. If possible, the soil, dust, sewage, and vegetable samples were also collected at least three samples per farm.

In order to examine the spread of mobile tigecycline resistance gene *tet*(X4), samples and clinical isolates were screened from four tertiary care hospitals and 63 intensive animal farms (pig farms, n=41; chicken houses, n=22) in 16 provinces or municipalities in China (Fig. 1). The four hospitals are located in Guangdong (hospital A and B), Jiangsu (hospital C), and Zhejiang (hospital D) provinces, respectively. Pig farms and chicken houses are distributed in four geographic regions of China (northeast, southeast, west, and central China) and cover most livestock breeding areas in China. Briefly, a total of 4189 samples from the animal sector were collected from May 2015 to May 2018 (Supplementary Table 2) and subjected to selection on MacConkey agar (MAC) plates containing tigecycline (2 mg/L). The selected tigecycline-nonsusceptible isolates were then subjected to *tet*(X4) screening. In addition, 299 human specimens from hospital A collected in the same period and a random collection of 720 *E. coli* clinical isolates from hospitals B, C, and D were also screened as described above (Supplementary Table 2).

Porcine *E. coli* LHM10–1 was selected as the donor strain while laboratory strains (*E. coli* C600 [streptomycin-resistance], *Salmonella Typhimurium* ATCC 14028 [rifampin-resistance], and *K. pneumoniae* ATCC 700603 [rifampin-resistance]), and clinical MDR strains (*E. coli* 1314 carrying $bla_{\text{NDM-5}}$ [ST167] and *K. pneumoniae* 1332 carrying $bla_{\text{KPC-2}}$ [ST11]) were chosen as recipient strains for conjugation and plasmid stability tests. Porcine *E. coli* STB20–1 carrying a single plasmid [*tet*(X4)-positive] was selected for the *in vivo* infection model.

Whole genome sequencing (WGS)

Genomic DNA was extracted from *E. coli* LHM10–1 using a TIANamp Bacteria DNA kit (Tiangen, Beijing, China), followed by genomic DNA sequencing with a MiSeq platform (Huayin Health, Guangzhou, China). To obtain the complete plasmid sequences, *E. coli* LHM10–1 was also subjected to single-molecule real-time (SMRT) sequencing with a PacBio RSII system (Nextomics, Wuhan, China). In addition, another *tet*(X4)-positive *E. coli* strain G3X16–2 was completely sequenced using a combined Illumina HiSeq and Nanopore sequencing approach (Nextomics, Wuhan, China). Genome assembly was performed with Unicycler version 0.4.1 (ref. 27) using a combination of short and long reads, followed by error correction by Pilon version 1.12 (ref. 28). The functional genes were annotated and classified using the RAST server[29]. The sequence types and plasmid replicon types were analyzed by the CGE server (https://cge.cbs.dtu.dk/services/). Visual representation of the genetic structure of *tet*(X4)-carrying plasmids was generated with DNAPlotter version 1.11 (ref. 30).

Subcloning experiments

To confirm the role of Tet(X4) in tigecycline resistance, the full-length open reading frame (ORF) of *tet*(X4) was amplified from genomic DNA of *E. coli* LHM10–1 with addition of an *EcoR*I restriction site at the 5′-end and a *Sal*I restriction site at the 3′-end (Supplementary Table 4). Both the purified PCR product and the plasmid vector pBAD24 containing the arabinose pBAD promoter were digested with restriction endonucleases *EcoR*I/*Sal*I and then ligated at 16 °C overnight following the manufacturer's instructions (New England Biolabs, Ipswich, MA). Subsequently, the recombinant plasmid pBAD24-*tet*(X4) was transformed into competent *E. coli* JM109 by heat shock and then subjected to susceptibility testing against different tetracyclines using a broth microdilution method.

Microbiological degradation assays

The activities of Tet(X4) on tetracycline antibiotics degradation were initially assessed by an agar well diffusion method[31]. Firstly, a suspension of the *E. coli* construct JM109+pBAD24-*tet*(X4) was made by suspending the culture from a Mueller-Hinton (MH) agar plate in 500 μl of MH broth containing 0.1% L-arabinose (OD_{600} adjusted as 2.0). The suspension was then inoculated with tetracycline (10 mg/L), oxytetracycline (20 mg/L), chlortetracycline (20 mg/L), doxycycline (10 mg/L), minocycline (10 mg/L), tigecycline (2.5 mg/L), and eravacycline (2.5 mg/L), respectively, and cultured at 37 °C for 8 hours. Meanwhile, 100 μl of the overnight culture (0.5 McFarland) of *Bacillus stearothermophilus* 7953 (tetracyclines-susceptible) was spread on a MH agar plate surface. A hole with 6 mm diameter was punched aseptically with a disposable hole puncher, and its bottom was sealed by a drop of heated liquid MH agar. Following centrifugation and filtration with a 0.22 μm filter, 20 μl of the supernatant from the JM109+pBAD24-*tet*(X4) coculture with different tetracycline antibiotics was added into the prepared agar hole and the plate was incubated at 60 °C for 16 hours to examine the zone of inhibition. The *E. coli* JM109+pBAD24 strain was used as a negative control and the medium containing only tetracyclines was acted as the blank control.

Quantifying the levels of tetracyclines via liquid chromatography-tandem mass spectrometry (LC-MS/MS)

To further examine if Tet(X4) was able to inactivate tetracyclines, the levels of tetracycline and eravacycline (tigecycline wasn't used because of its poor stability in growth media, even with the protection of 0.6 g/L ascorbic acid as previously described[32]) were quantified in growth media of *E. coli* JM109+pBAD24-*tet*(X4) by LC-MS/MS in sextuplicate, respectively[17]. For tetracycline quantification, *E. coli* JM109+pBAD24-*tet*(X4) was grown in 4 ml of M9 minimal media (1×M9 minimal salts, 2 mM $MgSO_4$, and 100 μM $CaCl_2$) containing 0.1% L-arabinose supplemented with 9 g/L glucose, 100 mg/L thiamine, 100 mg/L leucine, and 8 mg/L tetracycline. The strain was incubated at 37 °C with shaking at 200 rpm and protected from light for 16 hours. Subsequently, cells were pelleted, and the supernatant was passed through a 0.22 μm filter. The filtered supernatant was then diluted 10-fold and subjected to LC-MS/MS quantification. Similarly, the level of eravacycline degradation was also determined by incubation with eravacycline (2 mg/L) for 16 hours. The *E. coli* JM109+pBAD24 strain was used as a negative control. The linear ranges of standard

curves for tetracycline and eravacycline quantification were 25 to 800 ppb and 10 to 200 ppb, respectively, with r^2 values >0.995.

Mating experiments and plasmid stability testing

The transferability of *tet*(X4) in *E. coli* LHM10–1 was determined by filter mating using *E. coli* C600, *S. Typhimurium* ATCC 14028, *K. pneumoniae* ATCC 700603, *E. coli* 1314, and *K. pneumoniae* 1332 as recipient strains. Briefly, the donor and recipient strains were grown at log-phase in Luria-Bertani (LB) broth, and were then mixed at the donor to recipient ratio of 1:3 and applied to a 0.22 μm filter, followed by culture at 37 °C for 16 hours. The putative transconjugants were selected as follows. For *E. coli* C600 and *S. Typhimurium* ATCC 14028, they were selected by MAC plates containing tigecycline (4 mg/L) in combined with streptomycin (1000 mg/L) or rifampin (100 mg/L). 8 mg/L of tigecycline together with rifampin (100 mg/L) was used for the selection of the putative transconjugants in *K. pneumoniae* ATCC 700603, which has an MIC 4 mg/L for tigecycline. For *E. coli* 1314 and 1332, they were selected by MAC plates containing tigecycline (4 mg/L) and meropenem (4 mg/L). Transconjugants were further confirmed by PCR screening for *tet*(X4), Enterobacterial Repetitive Intergenic Consensus PCR (ERIC-PCR), and 16S rRNA gene sequencing with the primers in appendix (Supplementary Table 4). Transfer efficiency was calculated based on colony counts of the transconjugant and recipient cells in triplicate as previously reported[33].

Subsequently, the host *E. coli* LHM10–1 strain and its transconjugants were subjected to plasmid stability testing as previously described[34]. Briefly, three independent lineages of them were cultured overnight at 37 °C in 4 ml of antibiotic-free LB broth, respectively. Serial passaging of 4 μl of the overnight culture to 4 ml of LB broth was performed daily, yielding approximately 10 generations of growth per passage. For every 20 generations, samples were diluted and plated on LB agar plates. Then, 50 colonies from each lineage were screened on LB agar plates with or without tigecycline (4 mg/L) to determine the fraction of plasmid-containing cells. 8 μg/ml of tigecycline was used for the transconjugant generated with *K. pneumoniae* ATCC 700603. The loss of plasmids was confirmed by the *tet*(X4) target PCR and ERIC-PCR as mentioned above.

In addition, the mobilizing efficiency of different helper plasmids on IncQ1 plasmid pLHM10–1-p6 was determined by filter mating in triplicate. Firstly, donor strains containing pLHM10–1-p6 and a single putative helper plasmid were constructed. In brief, the IncQ1 plasmid pLHM10–1-p6 was transferred into *E. coli* DH5α by heat shock and the putative transformant (namely *E. coli* DH5α+ pLHM10–1-p6) was selected by LB agar plates containing 4 mg/L of tigecycline. Then, three laboratory *E. coli* strains Z6–2, H12–4, and HLJ179, which have been confirmed to carry *mcr-1*-positive lncX4, lncFII, and I2 plasmids, respectively, were used for filter mating with the recipient strain *E. coli* DH5α+ pLHM10–1-p6. The putative constructs were selected by LB agar plates containing tigecycline (4 mg/L) and colistin (2 mg/L). PCR-based replicon typing (PBRT)[35–37], S1-digested pulsed field gel electrophoresis (S1-PFGE)[4], and ERIC-PCR were conducted to confirm that only a single *mcr-1*-harboring helper plasmid was transferred into *E. coli* DH5α+ pLHM10–1-p6. Secondly, the pLHM10–1-p6-harboring *E. coli* DH5α strains with a single putative helper

plasmid (namely pZ6–2, pH12–4, or pHLJ179) were used as donors for conjugation, with *E. coli* C600 as the recipient strain, followed by selection of transconjugants with the LB agar plates containing tigecycline (4 mg/L) and streptomycin (1000 mg/L). The putative transconjugants and mobilizing efficiency were further determined as described above, respectively.

Screening for the presence of *tet*(X4) and *tet*(X4)-harboring plasmid like pLHM10–1-p6

All tigecycline-nonsusceptible isolates were screened for *tet*(X4) by PCR, followed by Sanger sequencing. The pLHM10–1-p6-like plasmid was also screened by overlapping PCRs targeting four overlapping regions (IncQ-1 [Δ*czcD*-*orf1*-ΔIS*CR2*-Δ*tet*(X4)], IncQ-2 [Δ*tet*(X4)-*catD*-IS*CR2*-Δ*orf2*], IncQ-3 [Δ*orf2*-*mobC*-*mobA*-*mobB*-*repB*-Δ*repF*], and IncQ-4 [*repF*-*repA*-*repC*-Δ*sul2*-*czcD*-Δ*orf1*]) on pLHM10–1-p6 (Supplementary Fig. 3). All primers used here were listed in appendix (Supplementary Table 4).

Antimicrobial susceptibility testing

MICs of ten antibiotics (tetracycline, amikacin, gentamicin, meropenem, cefoxitin, cefotaxime, fosfomycin, ciprofloxacin, sulfamethoxazole/trimethoprim, and florfenicol) for all *tet*(X4)-harboring strains were determined by agar dilution and interpreted according to the Clinical & Laboratory Standards Institute (CLSI) guidelines. MICs of tigecycline, eravacycline, and colistin were determined by broth microdilution. Especially, the breakpoint of tigecycline for *E. coli* strains was interpreted according to the FDA criteria (susceptible, ≤2 mg/L; intermediate, 4 mg/L; resistant, ≥8 mg/L), while the eravacycline was uninterpreted with no breakpoint. Colistin was interpreted in accordance with the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoint (susceptible, ≤2 mg/L; resistant, >2 mg/L). *E. coli* ATCC 25922 was served as a quality control strain for susceptibility testing.

Molecular typing

All *tet*(X4)-positive *E. coli* strains were classified by *Xba*I-digested (Takara, Dalian, China) PFGE according to the PulseNet protocol (http://www.pulsenetinternational.org/protocols/) using a CHEF Mapper® System (Bio-Rad, Hercules, CA, USA). PFGE patterns were compared using BioNumerics version 6.6 (Applied Maths Software) under appropriate optimization (1.5%) and tolerance (1.5%) settings, with a cutoff at 85% similarity to delineate PFGE clusters. Multilocus sequence typing (MLST) was performed by the primers and protocol specified in *E. coli* MLST database website (http://enterobase.warwick.ac.uk).

Murine thigh infection model

Female ICR mice (6–10 weeks old; Guangdong Medical Lab Animal Center, Guangzhou, China) were used in this experiment. Mice were maintained in accordance with the National Standards for Laboratory Animals in China (GB 14925–2010). All animal studies were conducted in accordance with SCAU Institutional Animal Welfare and Ethics guidelines. The animal use procedures were approved by the Animal Research Committees of SCAU. No specific statistical consideration was taken in determining the sample size for the animal experiments. Investigators were blinded as for group allocation when determining colony

forming unit (CFU). Meanwhile, we were able to cure the *tet*(X4)-carrying plasmid from wild-type *E. coli* STB20–1 [*tet*(X4)-positive] using sodium dodecyl sulfate (SDS) as previously described[10], to create STB20–1S [*tet*(X4)-negative], and thereby any difference caused by treatment will be most likely due to the presence of the plasmid only.

Prior to infection, mice were rendered neutropenic by injecting two doses of cyclophosphamide intraperitoneally on 4 days (150 mg/kg) and 1 day (100 mg/kg). In order to study the impact of the *tet*(X4)-carrying plasmid on efficacy of tigecycline, thigh infections were produced by injecting 0.1 ml of suspensions of *E. coli* STB20–1 or its *tet*(X4)-cured STB20–1S strain at 10^6 CFU/ml. At 2 hours post-infection, the mice were administered subcutaneously with a single dose of tigecycline at 50 mg/kg per 24 hours, which was approximately equivalent to the dosing regimen in humans given as 50 mg per 12 hours for 6 days[38,39]. Mice were humanely euthanized at 24 hours after treatment and thigh muscles were aseptically homogenized in 3 ml of saline. After serial dilutions, suspensions were quantitatively cultured on antibiotic-free LB agar plates for CFU enumeration.

Statistical analysis

Descriptive analyses on percentage (together with standard deviation) and prevalence were performed using functions provided in Excel 2007 (Microsoft Software). All statistical analyses (unpaired *t* test, two tailed; two-way ANOVA) were performed with GraphPad Prism 5 (GraphPad Software).

Data availability

The whole genome sequence data of *E. coli* LHM10–1 and G3X16–2 strains have been submitted to NCBI under the BioSample accession number SAMN11087649 and SAMN11180601, respectively. Extra data supporting the findings of this study are available from the corresponding authors upon reasonable request.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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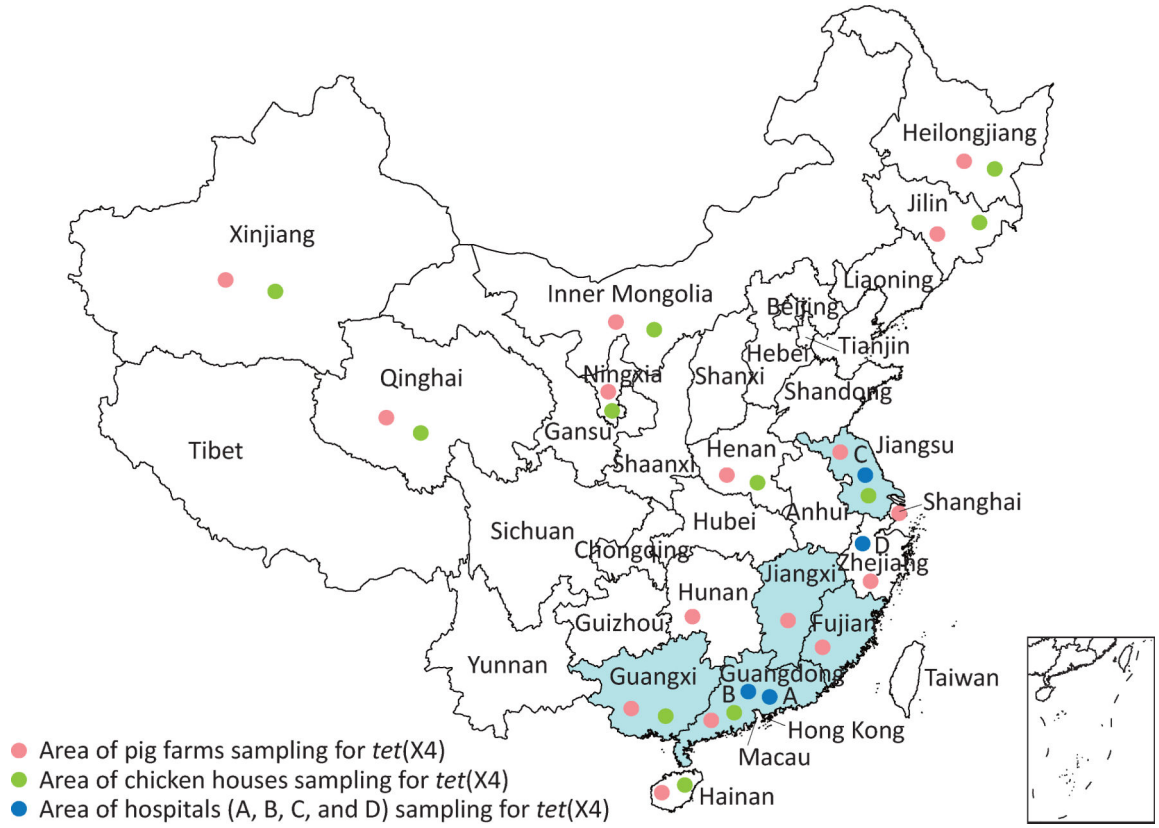


Figure 1 |. Map of *tet*(X4) sampling areas in China.

The *tet*(X4) sampling areas of pig farms, chicken houses, and hospitals (A, B, C, and D) are denoted with pink, green, and blue circles, respectively. The distribution of *tet*(X4)-positive *E. coli* strains in China is shaded in light blue.

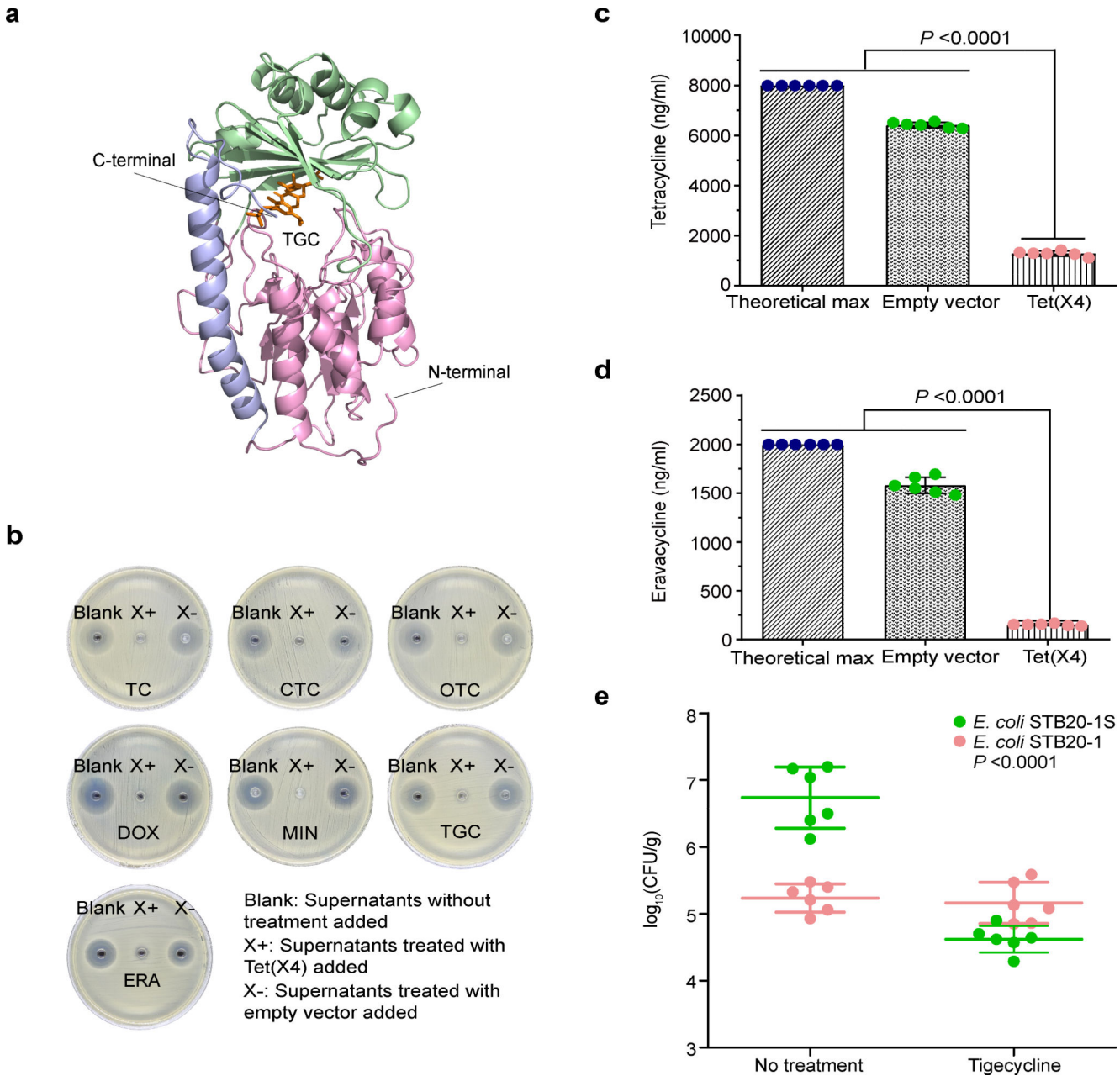


Figure 2 |. The activity of Tet(X4) on tetracyclines *in vitro* and *in vivo*.

a, Homology modelling of the tetracyclines-inactivating protein Tet(X4). The modelling of Tet(X4) is based on published tigecycline/Tet(X) complex (PDB accession number 4A6N[40]) using the online server Phyre2 (ref. 41) and AutoDock version 4.2.6 (ref. 42). The substrate binding domain (light green), FAD binding domain (pink), C-terminal helix (light blue), and tigecycline (orange) are displayed. The C-terminal and N-terminal are marked in black characters. **b,** Microbiological degradation assays. The activity of *tet*(X4) on tetracycline antibiotics degradation is evaluated by measuring the changes of inhibition zones after the addition of supernatant from different cocultures. The experiments are performed in triplicate and repeated three times with similar results. Blank, the supernatant without

treatment (only containing tetracyclines) is added; X+, the supernatant from the coculture incubated with the *tet*(X4) construct, namely *E. coli* JM109+pBAD24-*tet*(X4), is added; X-, the supernatant from the coculture incubated with *E. coli* JM109 containing the empty vector pBAD24 alone is added; TC, tetracycline; CTC, chlortetracycline; OTC, oxytetracycline; DOX, doxycycline; MIN, minocycline; TGC, tigecycline; ERA, eravacycline. **c-d,** The levels of tetracycline (**c**) and eravacycline (**d**) degradation by Tet(X4). Statistical analysis is conducted using unpaired and two sided *t* test. Individual values of biological replicates (n=6) are shown as dots, while the means (middle lines) and standard deviations are displayed as error bars. Theoretical max indicates the initial tetracycline or eravacycline concentration in the medium prior to inoculation. **e,** *In vivo* effects of tigecycline treatment (50 mg/kg per 24 hours) in a murine thigh model. *P* value is calculated by a two-way ANOVA test. Individual values of animals (n=6) are shown as dots, while the means (middle lines) and standard deviations are displayed as error bars.

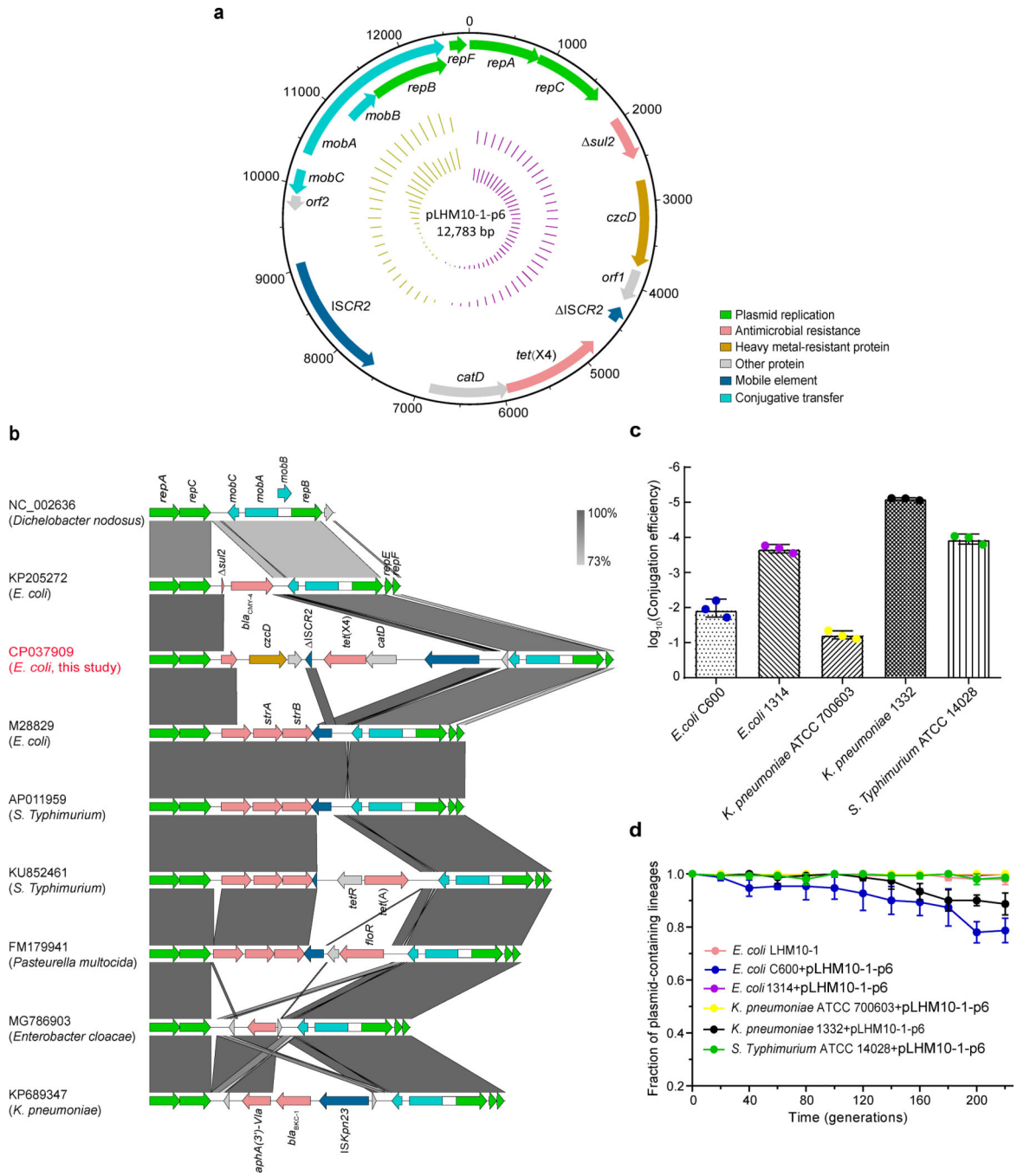
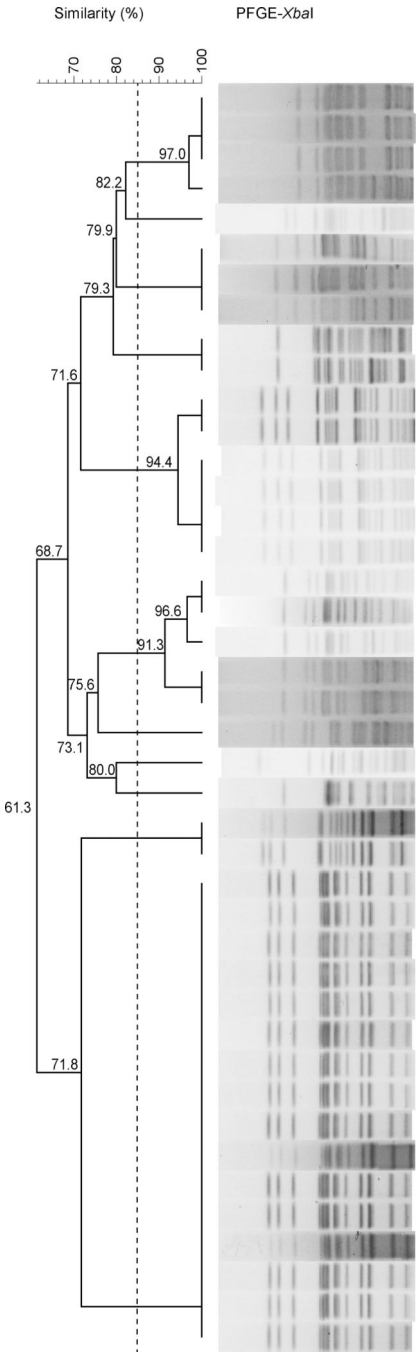


Figure 3 |. Characteristics of the IncQ1 *tet*(X4)-harboring plasmid pLHM10–1-p6.

a, Structure of the index plasmid pLHM10–1-p6. GC skew and GC content are indicated from the inside out. Positions and transcriptional directions of the predicted ORFs are denoted with arrows. Genes associated with the plasmid replication, antimicrobial resistance, heavy metal resistance, mobile element, and conjugative transfer are highlighted in green, pink, dark yellow, blue, and cyan, respectively. Other genes are marked as grey arrows. **b,** Linear comparison of the representative IncQ1 plasmid sequences. Results of sequence alignment are generated with Easyfig version 2.1 (ref. 43). The arrows represent

the position and transcriptional direction of the ORFs. Regions of homology between 73% and 100% are marked by grey shading. **c,** Conjugation transfer efficiencies of the index plasmid pLHM10–1-p6 into *E. coli*, *K. pneumoniae*, and *S. Typhimurium* strains. Transfer efficiency is calculated based on colony counts of the transconjugant and recipient cells in triplicate, and all data points are displayed, along with mean and standard deviation, respectively. **d,** Plasmid stability experiment results. All experiments are conducted in triplicate. Error bars denote the means (middle lines) and standard deviations.



Isolate	Date	Province	Source	PFGE pattern	MLST	Resistance phenotype[b]
G3P11-1	16/08/2017	Guangxi	Pig	A	ST1196	TGC/TC/SXT/FFC/CTX/CS/CIP
G3X12-2	16/08/2017	Guangxi	Pig	A	ST1196	TGC/TC/SXT/FFC/CTX/CS/CIP
G3X16-2	16/08/2017	Guangxi	Pig	A	ST1196	TGC/TC/SXT/FFC/CTX/CS/CIP
G3P8-2	16/08/2017	Guangxi	Pig	A	ST1196	TGC/TC/SXT/FFC/CTX/CS/CIP
LHZ2-1	16/07/2017	Jiangxi	Pig	B	ST761	TGC/TC/SXT/FFC/CTX/GEN
XFYP18-1	15/07/2017	Fujian	Pig	C	ST8338	TGC/TC/SXT/FFC
XFYP7-1	15/07/2017	Fujian	Pig	C	ST8338	TGC/TC/SXT/FFC
XFYS1-2	15/07/2017	Fujian	Soil[a]	C	ST8338	TGC/TC/SXT/FFC
YSB3-1	27/07/2017	Guangdong	Pig	D	ST10	TGC/TC/SXT/FFC/CIP
YSB7-2	27/07/2017	Guangdong	Pig	D	ST10	TGC/TC/SXT/FFC/CIP
DDX6-3	14/07/2017	Fujian	Pig	E	ST515	TGC/TC/SXT/FFC/CTX/CS/CIP/GEN
LHS1-1	16/07/2017	Jiangxi	Soil[a]	E	ST515	TGC/TC/SXT/FFC/CTX/CS/CIP/GEN
DDBR1-3	14/07/2017	Fujian	Dust[a]	E	ST515	TGC/TC/SXT/FFC/CTX/CS/CIP/GEN
DDX16-1	14/07/2017	Fujian	Pig	E	ST515	TGC/TC/SXT/FFC/CTX/CS/CIP/GEN
LHM10-1	16/07/2017	Jiangxi	Pig	E	ST515	TGC/TC/SXT/FFC/CTX/CS/CIP/GEN
LHP6-1	16/07/2017	Jiangxi	Pig	E	ST515	TGC/TC/SXT/FFC/CTX/CIP/GEN
THP14-1	14/07/2017	Fujian	Pig	F	ST761	TGC/TC/SXT/FFC
THX8-2	14/07/2017	Fujian	Pig	F	ST761	TGC/TC/SXT/FFC
STB20-1	08/05/2017	Jiangsu	Pig	F	ST761	TGC/TC/SXT/FFC
THX2-1	14/07/2017	Fujian	Pig	F	ST761	TGC/TC/SXT/FFC
THX3-2	14/07/2017	Fujian	Pig	F	ST761	TGC/TC/SXT/FFC/GEN
GQ65-1	14/11/2017	Guangxi	Chicken	G	ST10	TGC/TC/SXT/FFC
LHZ4-1	16/07/2017	Jiangxi	Pig	H	ST871	TGC/TC/SXT/FFC/CIP
YSP4-1	27/07/2017	Guangdong	Pig	I	ST101	TGC/TC/SXT/FFC
HCP21-2	27/07/2017	Guangdong	Pig	J	ST542	TGC/TC/SXT/FFC/CIP/GEN
YSP8-1	27/07/2017	Guangdong	Pig	J	ST542	TGC/TC/SXT/FFC/CTX/CIP
DDPR2-2	14/07/2017	Fujian	Dust[a]	K	ST8302	TGC/TC/SXT/FFC
GDXB17-2	20/06/2017	Jiangxi	Pig	K	ST8302	TGC/TC/SXT/FFC
JS100	09/07/2017	Jiangsu	Chicken	K	ST8302	TGC/TC/SXT/FFC
JS104-2	09/07/2017	Jiangsu	Chicken	K	ST8302	TGC/TC/SXT/FFC
JS105-1	09/07/2017	Jiangsu	Chicken	K	ST8302	TGC/TC/SXT/FFC
JS116-2	09/07/2017	Jiangsu	Chicken	K	ST8302	TGC/TC/SXT/FFC
JS77-1	09/07/2017	Jiangsu	Chicken	K	ST8302	TGC/TC/SXT/FFC
JS78-1	09/07/2017	Jiangsu	Chicken	K	ST8302	TGC/TC/SXT/FFC
JS80-1	09/07/2017	Jiangsu	Chicken	K	ST8302	TGC/TC/SXT/FFC
XDBN7-1	20/07/2017	Guangdong	Pig	K	ST8302	TGC/TC/SXT/FFC
XDP15-1-1	20/07/2017	Guangdong	Pig	K	ST8302	TGC/TC/SXT/FFC
XDP6-1-2	20/07/2017	Guangdong	Pig	K	ST8302	TGC/TC/SXT/FFC
XDP9-1	20/07/2017	Guangdong	Pig	K	ST8302	TGC/TC/SXT/FFC
XDXN3-3-1	20/07/2017	Guangdong	Pig	K	ST8302	TGC/TC/SXT/FFC
XDXN5-2-1	20/07/2017	Guangdong	Pig	K	ST8302	TGC/TC/SXT/FFC
XDXN8-1-1	20/07/2017	Guangdong	Pig	K	ST8302	TGC/TC/SXT/FFC/GEN

Figure 4 |. *Xba*I-PFGE dendrogram and details about *tet*(X4)-positive *E. coli* isolates.

The PFGE assay is conducted successfully for once according to the standard protocol, and then used for the following analysis. The full gel images have also been provided in the supplementary material. PFGE patterns with a cutoff at 85% similarity (the dotted line) are considered to the same PFGE cluster, and indicated as groups A-K, respectively. *E. coli* strains carrying pLHM10–1-p6-like plasmid are underlined. [a]These strains are isolated from environmental samples in pig farms. [b]TGC, tigecycline; TC, tetracycline; SXT,

sulfamethoxazole/trimethoprim; FFC, florfenicol; CTX, cefotaxime; CS, colistin; CIP, ciprofloxacin; GEN, gentamicin.