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Short Communication

Limited distribution and mechanism of the TetX4 tetracycline resistance enzyme

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Tetracyclines, a group of antimicrobial agents targeting protein synthesis, have been extensively used in agricultural, veterinary, and clinic settings [1]. The sporadic rise in tetracycline resistance is partially due to the occurrence of efflux pumps and/or ribosome protection [1,2]. The growing body of tetracycline-inactivating enzymes (e.g., TetX (X1 to X4) [3,4] and Tet (47 to 55) [5]) represents an alternative mechanism for tetracycline resistance, and threatens the renewed interest of tigecycline as a last-resort defense against lethal infections with carbapenem-resistant Enterobacteriaceae and *Acinetobacter* species. The TetX-type enzyme belongs to the family of flavin-dependent monooxygenases that degrade a wide range of tetracycline derivatives, and consequently confer phenotypic resistance [6]. The prototypical version of TetX, is initially detected as a cryptic genetic determinant within transposons of R plasmid from the obligate anaerobe *Bacteroides fragilis* [7], and then determined to be a NADP-requiring oxidoreductase using the aerobic growth system of *Escherichia coli* [4]. Intriguingly, two additional *tetX* loci, namely *tetX1* and *tetX2*, are also identified from the Tn*4351* (Tn*4400*) conjugative transposons exclusively in *Bacteriodes* [3]. The third one, *tetX3* (Acc. no.: AB097942) is found in the opportunistic pathogen *Pseudomonas aeruginosa*, in 2002. With an exception of TetX1, all the other TetX members consistently display high level of identity (84.39% to 99.48%, in Fig. S1 online). In particular, the substitution of only two amino acids (K94E & M360I) is present in TetX2 when compared with the original one TetX (to relieve confusion, we prefer to rename it as TetX0). However, the structure-function relationship of a given TetX enzyme remains elusive.

On May 27th, 2019, Wang and coworkers reported two new *tetX* variants (*tetX3* and *tetX4*) conferring high-level tigecycline resistance [8]. The un-typable plasmid p34AB carrying *tetX3* (277,384 bp) is discovered in *Acinetobacter baumannii* strain 34AB, whereas the IncHI2-type, *tetX4*-harboring plasmid p48EC (170,312 bp) is detected in the *E. coli* strain 47EC [8]. It raises the possibility that the plasmid-aided spread of tigecycline resistance across benign and pathogenic species within Enterobacteriaceae. Linear alignment of *tetX4*-containing plasmids and/or contigs elucidates a relatively-conserved cassette of "*catD-tetX4*-IS*CR2* (ΔIS*CR2*)" (Fig. 1a). To our surprise, pG3X16-2-p2 (Acc. no.: CP038140) even harbors four tandem cassettes of "IS*CR2-catD-tetX4*-IS*CR2*". Together, these results suggest that the acquisition and spread of *tetX4* into diversified plasmids with multiple replicon types proceeds via genetic events of IS*CR2*-mediated translocation.

Though that an IS*CR2* transposon element is neighboring with *tetX4*, database mining with *tetX4* as a sequence probe, returns only eight hits (Fig. 1a). In brief, only four plasmids with full genome sequence are shown to harbor *tetX4* (Fig. 1a), which separately includes an IncQ1-type plasmid, pLHM10-1 (12,783 bp; Acc. no.: CP037909), the IncFIB-like plasmid, p47EC (170,312 bp; Acc. no.: MK134376), and two chimera plasmids, pYSP8-1 (IncX4-IncHI1A-IncHI1B-IncFIA; 239,588 bp; Acc. no.: CP037911) and pG3X16-2-3 (IncX1-IncFIA-IncFIB; 138,950 bp; Acc. no.: CP038140). Unlike that the four *tetX4*-harboring plasmids are restricted to swine/chicken rectal swabs in China, all the other four *tetX4*-containing contigs (deposited in 2015 to 2017) correlate to bacterial isolates exclusively in Thailand. Except that *Shigella flexneri* (Acc. no.: NIYR01000137) is originated from beef, the remaining three isolates are collected from human rectal swabs (Fig. 1a). Namely, they are *Klebsiella pneumoniae* (Acc. no.: NQBP01000050), *S. boydii* (Acc. no.: NQBY01000073), and *S. sonnei* (Acc. no.: NQAI01000053). Similar to those of p47EC (Acc. no.: IMK134376) and pG3X16-2-3 (Acc. no.: CP038140), the *tetX4*-bearing

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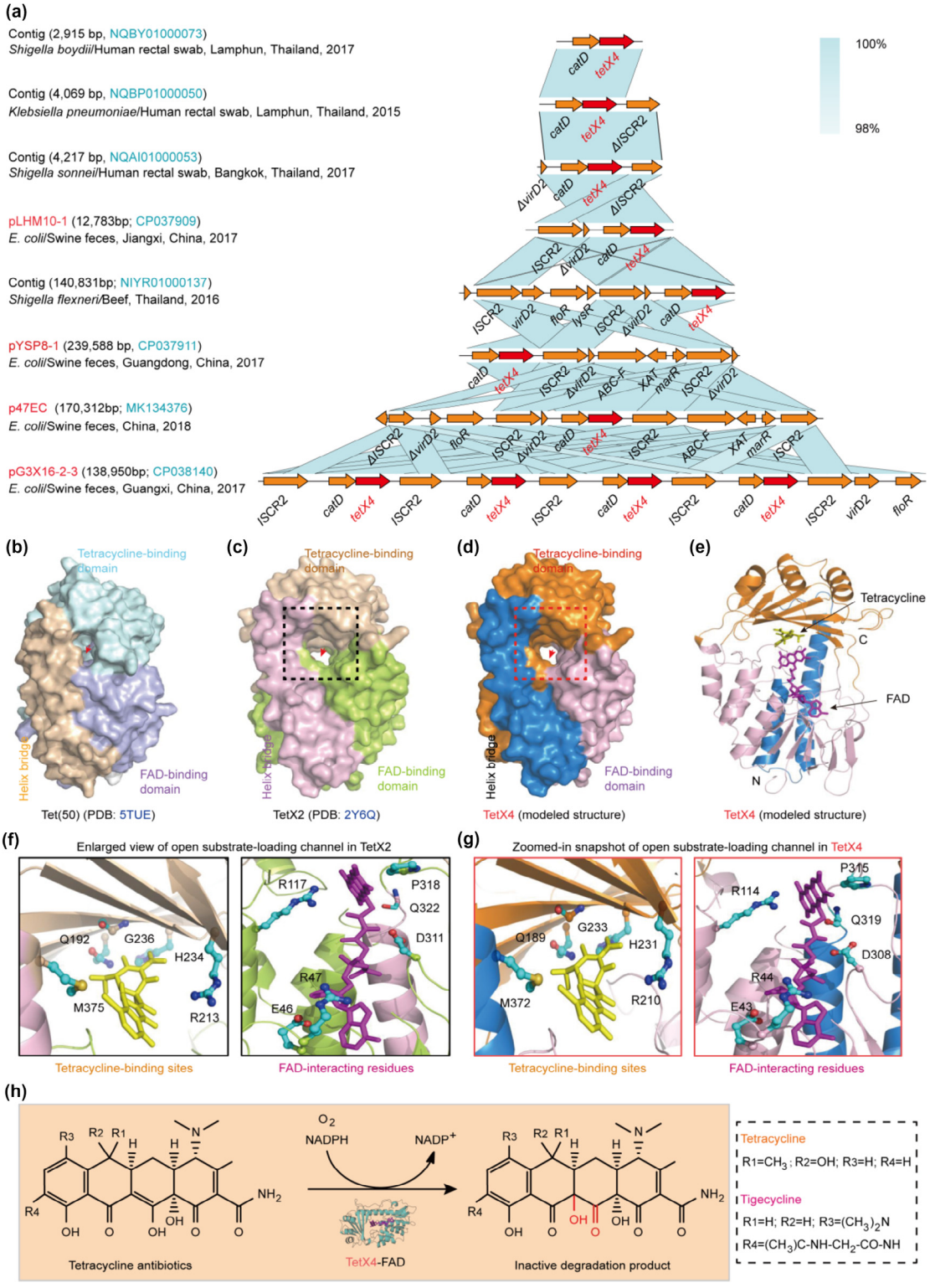

Fig. 1. Limited distribution and mechanism of the TetX4 tetracycline resistance enzyme. (a) Alignment for genetic context of *tetX4*-harboring plasmids and/or contigs. As of formulating this manuscript, only four *tetX4*-bearing plasmids with known genome sequences are deposited into GenBank. *In silico* searching in NCBI database returns us four additional *tetX4*-carrying contigs. Colored arrows indicate ORFs and the shaded region depicts sequence similarity. The *tetX4* resistance gene is highlighted in red. (b) Surface representation of Tet50 (PDB: 5TUE), a newly-identified member of the tetracycline-inactivating enzyme family. (c) Surface structure of TetX2 (PDB: 2Y6Q) carrying an open substrate-loading channel. (d) Surface illustration of the modeled TetX4 structure having similar architecture. The open substrate-loading channel is indicated with a dashed square. (e) Ribbon depiction of the modeled TetX4 structure. In general, each of Tet enzyme consists of a tetracycline-binding domain, which is connected by a helix bridge with a FAD-binding domain (in b-e). FAD, tetracycline, and the three distinct motifs of Tet enzymes are highlighted appropriately. (f) An enlarged view of open substrate-loading channel in TetX2 with an accessibility of the tetracycline substrate and FAD cofactor. (g) Structural snapshot of the putative open substrate-loading channel in TetX4. As for tetracycline-binding cavity in TetX2, five essential residues are required, namely Q192, R213, H234, G236, and M375 (in f). Equivalently, the five amino acids of TetX4 include Q189, R210, H231, G233, and M372, respectively (in g). In total, six residues (E46, R47, R117, D311, P318, and Q322) are implicated into FAD occupation by TetX2 (in f). Similarly, TetX4 (in g) also contains the conserved amino acids (E43, R44, R114, D308, P315, and Q319). (h) A suggestive TetX4-catalyzed reaction for enzymatic tetracycline (tigecycline) inactivation. Chemical structure of compound is generated with ChemDraw. FAD, flavin adenine dinucleotide; N, N-terminus; C, C-terminus.

contig (140,831 bp) from *S. flexneri* (Acc. no.: NIYR01000137) also encodes a florfenicol resistance gene *floR* (Fig. 1a). This suggests that IS*CR2* might facilitate the translocation of resistance genes other than *tetX4*.

To further probe its prevalence in clinical/ swine isolates, we develop a sensitive *tetX4* (*tetX5*)-specific molecular detection method (Fig. S2a online) and screen 1200 nonredundant bacterial samples (Table S1 online). It consists of 496 clinical isolates (2 *Salmonella enterica*, 32 *A. baumannii*, 96 *E. coli*, 364 *K. pneumoniae*, 1 *Enterobacter cloacae*, and 1 *Proteus mirabilis*) and 704 swine *E. coli* isolates of swine fecal swabs (346 isolates from Guizhou Province and 358 isolates in Guangdong Province) (Table S1 online). However, none is positive for *tetX4* (*tetX5*) in our PCR screen (Fig. S2b online). It is generally consistent with the scenario that less than 1% carriage (over 4000 swine samples) of *tetX4* in Enterobacteriaceae is recently reported by Sun et al. [9]. As for the limited distribution of *tetX4*, it seems unusual, but not without any precedent in that the mobile colistin resistance gene *mcr-1* is also detected in clinical sector at less than 1% percentage. In addition, the nomenclature of *tetX3* seems to be redundant/duplicated, and thus recommended to be renamed as *tetX5* (Figs. S1 and S3 online). Bacterial viability assays indicate that the expression of plasmid-borne both *tetX4* and *tetX5* allows the recipient *E. coli* strain JM109 to grow on LBA plates supplemented with 8–16 μg/mL of tigecycline (Fig. S3 online). The observation that an intermediate level of tigecycline resistance is conferred by TetX4 and TetX5 argues an over-statement of Wang and coworkers [8]. It might suggest the species/strains-dependence of TetX type tigecycline resistance, though we can not rule out the possibility of the unstable/degraded tigecycline used in their assays.

Despite that they are phylogenetically divergent, these tetracycline-inactivating enzymes display unexpectedly-paralleled structural architecture (Fig. 1b–d). Among them, TetX2 is the first one whose structure is decoded at 2.1 Å resolution [10]. A unique helix acts as a bridge to connect the tetracycline-binding domain and the FAD-interacting domain (Fig. 1c). Because that it has only ~20% identity to TetX2, Tet50 can not be assigned to the family of tetracycline destructases. However, crystal structure of Tet50 reveals its overall architecture analogous to that of TetX2 (Fig. 1b) [5]. More intriguingly, crystallographic study of both Tet50 and TetX2 elucidates an open substrate-loading channel (Fig. 1b and c). A similar scenario is also visualized in the modeled structure of TetX4 (Fig. 1d). Molecular docking of TetX4 further illustrates this suggestive cavity for the successive behaviors (entry, binding, and then exit) of FAD cofactor and tetracycline substrate (Fig. 1e). Consistent with that of TetX2 (Fig. 1f), the substrate-loading channel in TetX4 also can be divided into two unique motifs: tetracycline-binding motif and FAD-interacting motif (Fig. 1g). Five conserved residues are implicated into the interplay between TetX2 and tetracycline, including Q192, R213, H234, G236, and M375, respectively (Fig. 1f). The equivalent amino acids in TetX4 separately correspond to Q189, R210, H231, G233, and M372 (Fig. 1g). Similarly, the FAD cofactor-recognizing motif requires six residues (E46, R47, R117, D311, P318 & Q322 for TetX2, seen in Fig. 1f; E43, R44, R114, D308, P315 & Q319 for TetX4, seen in Fig. 1g).

This constitutes a paradigm that the three Tet isoenzymes (Tet50, TetX2, and TetX4) functionally unify within the family of tetracycline resistance proteins. We thus propose a working model for TetX4 enzymatic action, in which FAD is dependent, and oxygen is required prior to the degradation of tetracycline and its derivatives, like tigecycline (Fig. 1h). To further consolidate the structure-function relationship of the two substrate-interacting cavities, we performed site-directed mutagenesis of *tetX4*. As a result, we generated 11 derivatives of *tetX4* carrying a single point-mutations (5 mutants (Q189A, R210A, H231A, G233A & M372A) for tetracycline-binding cavity in Fig. S4a (online) and 6 mutants (E43A, R44A, R114A, D308A, P315A & Q319A) for FAD-interacting cavity in Fig. S4b (online)). Among the five residues involved in tetracycline-binding mutants, the alanine substitution of both H231 and M372 can reduce its ability of conferring tigecycline resistance in *E. coli* (Fig. S4a online). By contrast, 3 of the six FAD-interactive residues are found to have essential roles in the phenotypic tigecycline resistance. Namely, it refers to E43, R114, and D308 (Fig. S4b online). In particular, the mutant of TetX4 (D308A) only render the recipient *E. coli* MG1655 resistant to tigecycline at the level of 4.0 μg/mL (slightly higher than the cut-off of 2.0 μg/mL in the negative-control strain MG1655, but significantly lower than 32.0 μg/mL as for its wild-type, Fig. S4b online). Evidently, our results constitute a functional proof for genetic determinants required for full function of the TetX family enzymes, such as TetX4.

In summary, the discovery of two new variants (TetX4 and TetX5) furthers our mechanistic understanding tigecycline resistance caused by a growing body of tetracycline-inactivating enzymes. In spite of the overstated prevalence of such variants in environmental and clinic sectors occurrence, it does constitute a potential risk of plasmid-mediated dissemination of tigecycline resistance globally. Therefore, it necessitates the prevention, monitor, and control of resistance to tigecycline, an ultimate line of defense antibiotics, through an introduction of *tetX4* (and/or *tetX5*) into our routine approach of "one health" (environmental/animal/human sectors).

Conflict of interest

The authors declare that they have no conflict of interest.

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Author contributions

YF formulated this project; YX, LL, and YF performed experiments; YF, YX, LL, and JS analyzed the data and prepared figures; YF drafted this manuscript.

Appendix A. Supplementary material

Supplementary material to this article can be found online at https://doi.org/10.1016/j.scib.2019.08.024.

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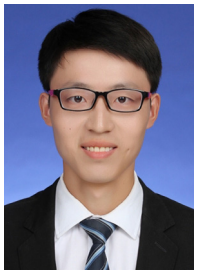

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