A Novel Transferable Resistance-Nodulation-Division Pump Gene Cluster, *tmexCD2-toprJ2*, Confers Tigecycline Resistance in *Raoultella ornithinolytica*

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ABSTRACT We recently identified a novel plasmid-mediated resistance-nodulation-division (RND)-type efflux pump gene cluster, *tmexCD1-toprJ1*, in *Klebsiella pneumoniae* that conferred resistance to multiple antimicrobials, including tigecycline. While homologs of *tmexCD1-toprJ1* were found encoded in many other bacterial species in GenBank, their functions and transfer mechanisms remain unknown. This study identified another mobile gene cluster, *tmexCD2-toprJ2*, co-occurring on both a plasmid (pHNNC189-2) and the chromosome of a clinical *Raoultella ornithinolytica* isolate, strain NC189, producing KPC-2, NDM-1, and RmtC. *tmexCD2-toprJ2* shares high similarity at the nucleotide level with *tmexCD1-toprJ1*, with 98.02%, 96.75%, and 99.93% identities to *tmexC1*, *tmexD1*, and *toprJ1*, respectively. Phylogenetic analysis revealed that *tmexCD2-toprJ2* may have originated from the chromosome of a *Pseudomonas* species. The expression of *tmexCD2-toprJ2* in an *Escherichia coli* strain resulted in an 8-fold increase in the tigecycline MIC and decreased susceptibility to other antimicrobials. Genetic context analyses demonstrated that *tmexCD2-toprJ2*, together with the adjacent hypothetical site-specific integrase genes, was possibly captured and mobilized by a XerD-like tyrosine recombinase system, forming a putative transposition unit (*xerD*-like-*int3*-like-*thf2-ybjD-umuD-ΔumuC1-int1*-like-*int2*-like-*hp1-hp2-tnfB2-ISBvi2-tmexCD2-toprJ2-ΔumuC1*), which was inserted into *umuC*-like genes in both the NC189 plasmid pHNNC189-2 and the chromosome. Since *tmexCD1-toprJ1* and *tmexCD2-toprJ2* could confer multidrug resistance, the spread of these gene clusters, associated with the new recombinase system, calls for more attention.

KEYWORDS plasmids, mobile genetic elements, *Pseudomonas* spp., site-specific integrase, efflux pumps, *Enterobacteriaceae*

Antibiotic resistance remains one of the biggest global public health challenges (1). Given the extensive distribution of carbapenem-resistant *Enterobacteriaceae* (CRE) and mobile colistin resistance (*mcr*) genes (2–4), antibiotic treatment options for bacterial infections have become increasingly limited (5). Tigecycline, derived and synthesized from tetracycline, has shown more-effective antimicrobial activity than tetracyclines (6, 7) and has been regarded as one of the last-resort treatment options for infections with carbapenem-resistant *Enterobacteriaceae* (8). Unfortunately, cases of infections caused by tigecycline-resistant *Enterobacteriaceae* have emerged and have been increasingly reported. Tigecycline resistance in various species of *Enterobacteriaceae* is mediated via multiple mechanisms, most of which involve chromosomal efflux pump overexpression

Citation Wang C-Z, Gao X, Yang Q-W, Lv L-C, Wan M, Yang J, Cai Z-P, Liu J-H. 2021. A novel transferable resistance-nodulation-division pump gene cluster, *tmexCD2-toprJ2*, confers tigecycline resistance in *Raoultella ornithinolytica*. Antimicrob Agents Chemother 65:e02229-20. <https://doi.org/10.1128/AAC.02229-20>.

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Received 22 October 2020

Returned for modification 3 December 2020

Accepted 8 January 2021

Accepted manuscript posted online 25 January 2021

Published 18 March 2021

caused by a variety of mutations in regulator genes, such as *ramR*, *acrR*, and *oqxR* (9–11). Bacterial efflux pumps, especially the resistance-nodulation-division (RND) superfamily, contribute to multidrug resistance (MDR) in bacteria owing to their ability to transport a wide variety of antibiotics (12). Some of these chromosomal RND pump genes could be horizontally transferred to other bacteria via mobile genetic elements (13, 14).

Recently, we reported for the first time the emergence of a plasmid-encoded RND-type pump gene cluster, *tmexCD1-toprJ1*, in *Klebsiella pneumoniae* isolates from animals, foods, and humans in China (15). *tmexCD1-toprJ1* exhibits a broad substrate spectrum, including glycolcyclines (tigecycline and eravacycline), tetracyclines, quinolones, cephalosporins, and aminoglycosides (15, 16). It is closely related to chromosomal *mexCD-oprJ* of *Pseudomonas aeruginosa* and is speculated to have originated from the chromosome of a *Pseudomonas* species. A series of *tmexCD1-toprJ1*-like sequences, deposited in GenBank, have been found in the plasmids of several bacterial species from multiple countries, implying that chromosomal *mexCD-oprJ*-like gene clusters were commonly captured by mobile elements (15); however, whether they could confer resistance to tigecycline and other antimicrobials remains unclear.

Thus, in this study, we identified and characterized another transferable *mexCD-oprJ*-like gene cluster, designated *tmexCD2-toprJ2*, which was carried by both the chromosome and a plasmid in a *Raoultella ornithinolytica* strain obtained from a patient in China.

RESULTS AND DISCUSSION

Identification of a novel gene cluster, *tnfxB2-tmexCD2-toprJ2*, in *R. ornithinolytica* strain NC189. *R. ornithinolytica* strain NC189 showed resistance to most antimicrobial agents, including imipenem and tigecycline, but susceptibility to colistin and ciprofloxacin (Table 1; see also Table S1 in the supplemental material). However, in the presence of the efflux pump inhibitor 1-(1-naphthylmethyl)-piperazine (NMP), it showed a 16- to 64-fold decrease in the MICs of tetracycline antibiotics, especially tigecycline (Table 1). The hybrid of Illumina and MinION reads of strain NC189 revealed that the strain harbored a 5,496,048-bp chromosome genome and five plasmids: pHNNC189-1, pHNNC189-2, pHNNC189-3, pHNNC189-4, and pHNNC189-5 (Table 2). Moreover, NC189 carried nine known antibiotic resistance genes (ARGs), including *bla*_{NDM-1}, *bla*_{KPC-2}, and *rmtC*. To the best of our knowledge, the coexistence of *bla*_{NDM-1} and *bla*_{KPC-2} and the presence of the 16S rRNA methyltransferase RmtC in *R. ornithinolytica* were observed for the first time. Interestingly, a novel *tnfxB-mexCD-oprJ*-like gene cluster, located on both the NC189 chromosome and plasmid pHNNC189-2, was identified. Compared with *tnfxB1-tmexCD1-toprJ1*, this cluster showed high sequence identities of 99.82%, 98.02%, 96.75%, and 99.93% to *tnfxB1*, *tmexC1*, *tmexD1*, and *toprJ1*, respectively, at the nucleotide level. The proteins encoded by this cluster showed 99.45%, 97.67%, 97.61%, and 99.79% amino acid identity to TNfxB1, TMexC1, TMexD1, and ToprJ1, respectively (Fig. S1 in the supplemental material). Therefore, this novel gene cluster was designated *tnfxB2-tmexCD2-toprJ2*.

To verify the role of *tmexCD2-toprJ2*, two recombinant plasmids, pHSG575-*tmexCD2-toprJ2* and pHSG575-*tmexCD2*, were constructed. Relative to those with the empty vector pHSG575, the recombinant strain carrying pHSG575-*tmexCD2-toprJ2*, showed 8-fold and 2- to 8-fold increases in the MICs of tigecycline and other antimicrobial agents (tetracyclines, quinolones, cephalosporins, florfenicol, and olaquinox), respectively (Table 1; also Table S1). However, in the presence of NMP, the MICs of tigecycline and other tetracyclines against *Escherichia coli* DH5 α carrying pHSG575-*tmexCD2-toprJ2* were decreased 32- to 64-fold, while MICs against *E. coli* DH5 α -pHSG575 decreased 4- to 8-fold. Further, these two recombinant strains showed the same levels of tigecycline and tetracycline MICs when exposed to NMP, suggesting that *tmexCD2-toprJ2* functions as an efflux pump system. In addition, 4- to 16-fold decreases in the MICs of tigecycline and tetracyclines were observed for *E. coli* DH5 α with pHSG575-*tmexCD2-toprJ2* in the presence of another efflux inhibitor, phenylalanine arginine β -naphthylamide (PA β N), exhibiting an effect lesser than that of NMP.

TABLE 1 MIC profiles for parental strains and transformants (mg/liter)^a

Strain	MIC (mg/liter) ^b													
	TIG ^b (+ NMP, + PA β N)	ERV (+ PA β N)	ERV (+ NMP, + PA β N)	DOX (+ NMP, + PA β N)	TET (+ NMP, + PA β N)	MIN	OXY	CTC	CTX	CAZ	CQM	FEP	CIP	OLA
<i>Raoultella ornithinolytica</i> NC189 ^c	4 (0.06, 1)	2 (0.06, 0.5)	2 (0.125, 0.5)	2 (0.125, 0.5)	2 (0.125, 1)	2	4	16	>128	>128	>128	16	0.03	16
<i>E. coli</i> DH5 α	0.25	0.5	1	1	0.5	2	2	4	0.03	0.125	0.03	0.03	0.002	1
<i>E. coli</i> DH5 α /pHSG575	0.25 (0.06, 0.125)	0.5 (0.06, 0.125)	1 (0.125, 0.25)	1 (0.125, 0.25)	0.5 (0.125, 0.125)	2	4	4	0.03	0.125	0.03	0.03	0.002	1
<i>E. coli</i> DH5 α /pHSG575-tmexCD2	0.5	1	2	2	1	2	4	8	0.06	0.125	0.125	0.06	0.008	4
<i>E. coli</i> DH5 α /pHSG575-tmexCD2-toprJ2	2 (0.06, 0.5)	2 (0.06, 0.5)	8 (0.125, 0.5)	8 (0.125, 0.5)	4 (0.125, 1)	4	8	16	0.125	0.25	0.25	0.125	0.015	4

^aAbbreviations: TIG, tigecycline; ERV, eravacycline; DOX, doxycycline; TET, tetracycline; MIN, minocycline; OXY, oxytetracycline; CTC, chlortetracycline; CTX, cefotaxime; CAZ, ceftazidime; CQM, ceftquinome; FEP, cefepime; CIP, ciprofloxacin; OLA, olaquinox; NMP, 1-(1-naphthylmethyl)-piperazine; PA β N, phenylalanine arginine β -naphthylamide.

^bMICs of tigecycline were determined by microdilution and those of other antibiotics by agar dilution. The MICs of tigecycline, eravacycline, doxycycline, and tetracycline were also determined with NMP (100 mg/liter) or PA β N (50 mg/liter).

^cThe imipenem MIC against NC189 was 4 mg/liter.

TABLE 2 Antimicrobial resistance genes and plasmids carried by *Raoultella ornithinolytica* NC189

Characteristic	Chromosome	Plasmid				
		pHNNC189-1	pHNNC189-2	pHNNC189-3	pHNNC189-4	pHNNC189-5
Size (bp)	5,496,048		196,122	124,023	99,419	51,221
Replicon type		N/A	FIB _K	FII _Y	FII	FIA
Gene(s) encoding resistance to:						
Aminoglycosides		<i>aac(3)-IId, aac(6')-Ib-cr</i>		<i>rmtC</i>		
β -Lactams	<i>bla_{PLA1a}</i>	<i>bla_{DHA-17}, bla_{TEM-1b7}, bla_{CTX-M-37}, bla_{OXA-1}</i>		<i>bla_{NDM-1}</i>	<i>bla_{KPC-2}</i>	
Fluoroquinolones	<i>oqxAB</i>	<i>qnrB4</i>				
Fosfomycin	<i>fosA</i>					
Sulfonamides		<i>sul1</i>		<i>sul1</i>		
Phenicol		<i>catB3</i>				
Rifampin		<i>arr3</i>				
Tetracyclines	<i>tmexCD2-toprJ2</i>		<i>tmexCD2-toprJ2</i>			

All these results suggested that *tmexCD2-toprJ2* indeed mediate resistance to multiple antimicrobials, especially tigecycline.

Characterization of plasmid pHNNC189-2 carrying *tmexCD2-toprJ2*. pHNNC189-2 is a 196,122-bp plasmid with an FIB_K-type replicon that contains 238 predicted open reading frames. However, this plasmid could not be transferred to recipient cells (*Escherichia coli* J53, C600, or DH5 α) by conjugation or transformation. Sequence analysis of pHNNC189-2 revealed that it contains two metal resistance gene clusters (the *silP-copG-cusA-cusB-cusF-cusC-cusR-cusS* cluster and the *copABCD* operon) and the *tnfxB2-tmexCD2-toprJ2* cluster but no other known resistance genes (Fig. 1A). Further, through a full plasmid BLAST query in GenBank, pHNNC189-2 was found to be closely related to the IncFIB_K plasmid pCAV1752-278 in *Klebsiella oxytoca* (CP018361.1), with highly similar plasmid backbones (Fig. 1A).

Phylogenetic analyses of TMexD2. Since the inner membrane protein plays a critical role in drug recognition and translocation, a phylogenetic tree of proteins homologous to the TMexD1 and TMexD2 proteins was constructed. Protein sequences could be classified into five major clusters: TMexD1, TMexD2, TMexD3 (another *tnfxB1-tmexCD1-toprJ1*-like gene cluster, named *tnfxB3-tmexCD3-toprJ3* here), TMexD3-like, and other MexD-like (Fig. 2). The phylogenetic tree showed that TMexD2 is closely related to TMexD1, and all TMexD proteins from various bacterial species shared a branching point (Fig. 2). Further analysis revealed that *tmexD* genes were located either on the plasmid or adjacent to mobile genetic elements, suggesting that *tmexD* genes were horizontally acquired but that *mexD*-like genes from *Pseudomonas* species in the rest of the branch were intrinsic. Furthermore, *tmexC2*, *tmexD2*, and *toprJ2* showed the highest nucleotide identities (82.27%, 91.13%, and 84.24%, respectively) to the efflux pump gene cluster genes located on the chromosome of *Pseudomonas* sp. strain LPH1 (GenBank accession no. CP017290.1). Therefore, *tnfxB2-tmexCD2-toprJ2* might originate from a *Pseudomonas* species.

Genetic context of *tnfxB2-tmexCD2-toprJ2*. Two identical *tnfxB2-tmexCD2-toprJ2* fragments were presented in the NC189 chromosome and plasmid pHNNC189-2. In these fragments, an ISBvi2 gene, belonging to the IS5 family transposase, was inserted into *tnfxB2*, resulting in the formation of a *tnfxB2-ISBvi2-tmexCD2-toprJ2* structure (Fig. 1B). The *tnfxB2-ISBvi2-tmexCD2-toprJ2* fragment was 100% identical to a fragment carried on the *Citrobacter freundii* plasmid pBKPC18-1 (GenBank accession no. CP022275.1) (from river sediment, Zhejiang, China) and 99.9% identical (one nucleotide substitution) to another cluster found in two *K. pneumoniae* strains (GenBank accession no. RXMT01000003.1 and RXMS01000085.1) (of human origin, Zhejiang, China). Additionally, a gene cluster on the chromosome of the *Aeromonas hydrophila* strain WCHAH045096 (GenBank accession no. CP028568.2) (from sewage, Sichuan, China) was also identical to the *tnfxB2-tmexCD2-toprJ2* cluster without the ISBvi2 insertion,

FIG 1 Plasmid structure and genetic context of *tmexCD2-toprJ2*. (A) Plasmid pHNNC189-2. The external ring represents the annotation of plasmid pHNNC189-2 (GenBank accession no. [MN175502](#)). Genes are color-coded for functional annotations (key at bottom left). The two internal rings represent a comparative analysis of pHNNC189-2 (red) with the IncFIB plasmid pCAV1752-278 (GenBank accession no. [CP018361.1](#)) (purple). The figure was constructed using BRIG. (B) Comparison of the genetic context of *tmexCD2-toprJ2* with those of closely related sequences. The extents and directions of genes are shown by arrows labeled with gene names. Black arrows, *tnfxB2-tmexCD2-toprJ2* and *tnfxB1-tmexCD1-toprJ1*-like gene clusters; pink arrows, *int* and *int*-like genes, predicted to encode site-specific integrases; purple arrows, *xerD*-like genes; light blue arrows, *umuC* and *umuD*; red arrows, *ISBiv2*; dark gray arrows and white arrows, genes that are unimportant in this study. Horizontal lines represent the plasmid backbone or chromosome. Regions of homology between 97% and 100% are shaded. The symbol Δ indicates that the gene is truncated.

showing only two nucleotide differences. Like the *tnfxB1-tmexCD1-toprJ1* cluster, *tnfxB2-ISBiv2-tmexCD2-toprJ2* was located adjacent to two genes that encoded two putative site-specific integrase genes (designated *int1-like* and *int2-like* here) and two hypothetical genes (designated *hp1* and *hp2* here) (Fig. 1B). These genes formed an assumed 16,849-bp transposition unit, *int1-like-int2-like-hp1-hp2-tnfxB2-ISBiv2-tmexCD2-toprJ2*, which was inserted into a *umuC*-like gene (designated *umuC1*) in both the NC189 plasmid pHNNC189-2 and the chromosome. The segment, including the two *int* and *hp* genes that were located upstream of *tnfxB2-ISBiv2-tmexCD2-toprJ2* in NC189, plasmid pBKPC18-1, and the chromosome of *A. hydrophila*, was identical to the region located upstream of *tnfxB1-tmexCD1-toprJ1* in the *K. pneumoniae* plasmid pHNAH81-1 (Fig. 1B). Furthermore, *tnfxB3-tmexCD3-toprJ3* was also located adjacent to the same *int1-like-int2-like-hp1-hp2* fragment that formed the putative mobile

FIG 1 Continued

module *int1-like-int2-like-hp1-hp2-tnxB3-tmexCD3-toprJ3*. Most of these putative transposition units (*int1-like-int2-like-hp1-hp2-tnxB-like-mexCD-like-oprJ-like* units) were inserted into the *umuC-like* genes located in the chromosomes or plasmids of various bacterial species, including *Pseudomonas* spp., *Aeromonas* spp., *K. pneumoniae*, *C. freundii*, and *R. ornithinolytica* (Fig. 1B). Therefore, this putative site-specific integrase system, "*int1-like-int2-like-hp1-hp2*," was frequently adjacent to *tnxB2-tmexCD2-toprJ2* and other (*tnxB1-tmexCD1-toprJ1*)-like gene clusters and have existed widely. At the same time, they lack homology with other known integrative mobile element units, such as XerC and XerD recombinases and class 1 integrons. These findings implied the emergence of a novel site-specific integration mechanism, which allowed the capture and transposition of the *tmexCD1-toprJ1*-like gene cluster. However, the biological functions of this putative integrase system require further elucidation.

Additionally, a XerD-like tyrosine recombinase gene, which shows 82% coverage and 26% amino acid similarity to *Shewanella oneidensis* XerD (NCBI Protein accession no. [AAN54024.1](#)), was identified upstream of the "*int1-like-int2-like-hp1-hp2-tnxB2-ISBvi2-tmexCD2-toprJ2*" module. XerD proteins, belonging to the tyrosine recombinase family, are involved in chromosome dimer resolution and DNA element integration and have been reported to mediate *tet39*, *bla_{OXA-58}*, and *bla_{OXA-24}* mobilization through the adjacent conserved XerC/XerD binding sites (17–19). The

Tree scale: 0.1

FIG 2 Phylogenetic tree of TMexD2 and TMexD2-like sequences. The amino acid sequences of 77 *tmexD2*-like genes were extracted from NCBI databases; then they were aligned and used to build a neighbor-joining tree using MEGA X. The result was validated with 1,000 bootstrap replicates and was visualized using iTOL. The letters around the outer ring indicate the locations of genes encoding the proteins as follows: C, chromosome; P, plasmid; CM, chromosome and adjacent to mobile genetic elements (suggesting that these are acquired rather than intrinsic genes); N, could not be identified as chromosome or plasmid. Information on the genera of strains carrying *tmexD2*-like genes, the groups of these genes, the countries where strains carrying these genes were isolated, and the sources of strains carrying these genes is color-coded in the outer ring, the backgrounds of the accession numbers, the inner ring, and the symbols in the middle of the branches, respectively.

xerD-like gene, together with adjacent elements, including another putative integrase gene (named *int3*-like here) and the “*int1*-like-*int2*-like-*hp1*-*hp2*-*tnfxB2*-*ISBvi2*-*tmexCD2*-*toprJ2*” module, likely formed a 29,060-bp putative transposition unit (*xerD*-like-*int3*-like-*thf2*-*ybjD*-*umuD*- Δ *umuC1*-*int1*-like-*int2*-like-*hp1*-*hp2*-*tnfxB2*-*ISBvi2*-*tmexCD2*-*toprJ2*- Δ *umuC1*), interrupting other *umuC*-like genes (designated *umuC2/3*) in both the NC189 chromosome and plasmid pHNNC189-2 (Fig. 1B). Similar genetic structures (*xerD*-like-*int3*-like-*thf2*-*ybjD*; 9,882-bp), carried by plasmids pWCGKP294-2 (GenBank accession no. [CP046614.1](#)) and pA324-IMP ([MF344566.1](#)) of *K. pneumoniae* strains isolated from humans in China, have been found in the GenBank database (20). Interestingly, pA324-IMP possessed an intact *umuC1* gene, which was 100% identical to the *umuC1* gene truncated by the “*int1*-like-*int2*-like-*hp1*-*hp2*-*tnfxB2*-*ISBvi2*-*tmexCD2*-*toprJ2*” unit in NC189. After the removal of this unit, the segments carried by the NC189 chromosome and plasmid pHNNC189-2 exhibited a genetic structure (*xerD*-like-*int3*-like-*thf2*-*ybjD*-*umuD*-*umuC1*) similar to that of p324-IMP, except for a deleted gene fragment that was located downstream of *ybjD* in pA324-IMP. This genetic structure of p324-IMP also seemingly constituted a transposition unit and was inserted into another *umuC*-like gene (*umuC4*) with the same insertion site (CTCTA CGC) as that of pHNNC189-2. The insertion site (CTCTA TGC) of the *xerD*-like-*int3*-like-*thf2*-*ybjD*-*umuD*- Δ *umuC1*-*int1*-like-*int2*-like-*hp1*-*hp2*-*tnfxB2*-*ISBvi2*-*tmexCD2*-*toprJ2*- Δ *umuC1* unit in *umuC3*

in the NC189 chromosome was very similar to that in pHNNC189-2 and p324-IMP (Fig. 2B), suggesting that this composite unit might be transferable between plasmids and chromosomes. Although similar genetic structures have been rarely found in the GenBank database, the XerD-like site-specific recombination system likely mediated the capture and mobilization of the transposition unit *int1*-like-*int2*-like-*hp1*-*hp2*-*tnfxB2*-*ISBvi2*-*tmexCD2*-*toprJ2* in NC189. However, no XerC/D-like conserved sites flanking *int1*-like-*int2*-like-*hp1*-*hp2*-*tnfxB2*-*ISBvi2*-*tmexCD2*-*toprJ2* were observed, suggesting that the XerD-like recombinase system in NC189 might act via another new recognition and recombinase mechanism. The underlying mechanism of this XerD-like recombinase system must be studied further.

MATERIALS AND METHODS

Bacterial strain. *R. ornithinolytica* strain NC189 was a clinical isolate recovered from a sputum sample from a 53-year-old female patient with a pulmonary infection and heart disease admitted at a major teaching hospital located in Jiangxi province in July 2018.

Antimicrobial susceptibility testing. MICs of antibiotics against isolates and transformants were determined by the agar dilution method (21). For colistin and tigecycline, MICs were measured using the microdilution method based on the Clinical and Laboratory Standards Institute (CLSI) guidelines (21). Tigecycline and tetracycline MICs were also measured in the presence of the efflux pump inhibitor NMP (100 mg/liter) or PA β N (50 mg/liter).

Conjugation and electroporation experiments. Conjugation experiments were performed with *E. coli* J53 and C600 as the recipient strains. Overnight cultures of the donor strain and J53 were mixed at a ratio of 1:1 in LB broth, and the mixture was then diluted and spread on a MacConkey agar plate containing tigecycline (2 mg/liter) and sodium azide (150 mg/liter) or streptomycin (2,000 mg/liter) for selecting transconjugants with J53 or C600 as the recipient strain, respectively. Electroporation transformation was performed based on a previous study (15), with *E. coli* DH5 α and *E. coli* BW25113 as the recipients. The transformants were screened on Luria-Bertani agar plates containing 2 mg/liter tigecycline, and PCR was performed to confirm the correct transformants carrying *tmexCD2*-*toprJ2*.

Genome sequencing and analysis. To characterize the genetic features of strain NC189, DNA fragments were extracted from overnight cultures by using the HiPure bacterial DNA kit (Magen). The complete genomic DNA data of NC189 were generated using a combination of the Nanopore MinION and Illumina HiSeq platforms. NC189 was subjected to whole-genome sequencing using the HiSeq platform (Illumina, San Diego, CA). Genome assembly was conducted with SPAdes, v3.13.0. Oxford Nanopore MinION sequencing was conducted to obtain the complete genome sequences of the strain by using the SQK-RBK004 sequencing kit and Flow Cell R9. A hybrid assembly of Illumina and Nanopore sequencing reads was constructed using Unicycler (22). Multilocus sequencing types, antimicrobial resistance genes (ARGs), and plasmid replicon types were identified by analysis by the Center for Genomic Epidemiology (<https://cge.cbs.dtu.dk/services/>). ISfinder (<https://www-is.biotoul.fr/>) and Galileo AMR (<https://galileoamr.arcbio.com/mara/>) were used to analyze the multidrug resistance regions and plasmids.

Gene cloning. DNA fragments that contained *tmexCD2*-*toprJ2* or *tmexCD2* were amplified using the primers listed in Table S2 in the supplemental material and were ligated to vector pHSG575 using a Seamless Assembly cloning kit (Clone Smarter Technologies Inc., Houston, TX, USA) to generate pHSG575-*tmexCD2*-*toprJ2* and pHSG575-*tmexCD2*. The *tmexCD2*-*toprJ2* genes within the recombinant plasmids have been resequenced, and the sequences were verified as correct. These two recombinant plasmids were then transformed into *E. coli* DH5 α . The MICs of antibiotics were determined as described above.

Phylogenetic analyses. To build a phylogenetic tree of proteins homologous to the TMexD1 and TMexD2 proteins, we collected 77 amino acid sequences from the nr NCBI database that show >80% amino acid identity to TMexD2. Multiple-sequence protein alignments were performed by ClustalW2, and a maximum-likelihood tree was built by MEGA X with 1,000 bootstrap replicates and was then annotated using iTOL (23–25).

Nucleotide sequence accession numbers. The complete sequences of the NC189 chromosome and plasmid pHNNC189-2 have been submitted to GenBank with accession numbers CP054471 and MN175502, respectively.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1.1 MB.

ACKNOWLEDGMENTS

This study was funded in part by the National Natural Science Foundation of China (grants 31625026 and 32002338), the Guangdong Special Support Program Innovation

Team (grant 2019BT02N054), the 111 Project (grant D20008), and the Innovation Team Project of Guangdong University (grant 2019KCXTD001).

Cheng-Zhen Wang, Xun Gao, and Qi-Wen Yang contributed equally to this work. Cheng-Zhen Wang is listed first because he was responsible for analyzing the genetic context of *tnfxB2-tmexCD2-toprJ2* and building the phylogenetic tree of TMexD2. He drafted most of the text. Xun Gao is listed second because she was responsible for confirming the *tmexCD2-toprJ2*-positive bacteria from clinical samples and sequenced the complete genome of strain NC189. She was involved in most of the experiments, including bacterial isolation and identification, gene cloning, MIC determination, and plasmid sequence analysis. Qi-Wen Yang is listed third because he was responsible for collecting human clinical strains and for clinical information.

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