

Emergence of plasmid-mediated tigecycline, β -lactam and florfenicol resistance genes *tet(X)*, *bla*_{OXA-347} and *floR* in *Riemerella anatipestifer* isolated in China

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ABSTRACT Bacterial antimicrobial resistance (AMR) continues to develop, with the horizontal transfer of antibiotic resistance genes (ARGs) through plasmids playing a major role. Recently, the antimicrobial resistance of *R. anatipestifer* has become increasingly severe, jeopardizing the development of the poultry industry. In this study, we used PromethION to determine the whole genome sequence of *R. anatipestifer* RCAD0416, a multidrug-resistant isolate from China. We detected a plasmid in the isolate. We named the plasmid pRCAD0416RA-1; the plasmid was 37356 bp in size with 36 putative open reading frames and included the *bla*_{OXA-347}, *floR*, *tet(X)*, *ermF*, *ereD*, and *AadS* resistance genes. Most resistance genes

might be obtained from *R. anatipestifer* HXb2. Mobile elements and *floR* might be transmitted by plasmid pB18–2 from *Acinetobacter indicus*, and the ICEP6gChn1 mobile elements can be transmitted from *Proteus genomosp.* The plasmid pRCAD0416RA-1 was transferred to *Escherichia coli* K-12 \times 7232 via electroporation. Subsequent antimicrobial sensitivity tests (AST) showed a noticeable levels of antimicrobial resistance to β -lactams (4–8 fold), tigecycline (8 fold), and florfenicol (8 fold). These types of antibiotics are in common clinical use. The purpose of this article is to elucidate the basic characteristics of pRCAD0416RA-1 and the level of resistance mediated by *bla*_{OXA-347}, *floR*, and *tet(X)*.

Key words: *bla*_{OXA}, *floR*, plasmid-mediated resistance, *Riemerella anatipestifer*, tigecycline, *tet(X)*

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INTRODUCTION

Riemerella anatipestifer is the main pathogen of avian septicemic and exudative diseases. Commonly used antimicrobial agents against these pathogens are tetracycline, β -lactams, fluoroquinolones, aminoglycosides, and quinolones. Reports on *R. anatipestifer* have shown antimicrobial resistance (AMR) worldwide (Zhong et al., 2009; Nhung et al., 2017). The resistance spectrum of *R. anatipestifer* is becoming increasingly wider, and multidrug resistance is extremely severe. The occurrence of AMR (like resistance to erythromycin, florfenicol, tigecycline) is mostly mediated by antibiotic

resistance genes (ARGs) (like *ermF*, *floR*, *tet(X)*). Acquired ARGs can be carried by plasmids from the environment and other bacteria (Agyare et al., 2018). Existing *R. anatipestifer* plasmid on NCBI, the database shows that most plasmids have been isolated from strains in Taiwan (Weng et al., 1999; Chen et al., 2010; Guo et al., 2017).

Florfenicol is an animal-specific antibacterial drug that plays an important role in veterinary clinical medication. Chloramphenicol/florfenicol efflux MFS transporter *floR* causes bacterial resistance to florfenicol and chloramphenicol. Currently, tigecycline is one of the last-resort antibiotics for treating bacterial infection. The rampant utilization of tetracycline has resulted in an increase in the tetracycline-resistant strains (Zhu et al., 2018). Tetracyclines modifying enzyme Tet(X) degrades the following compounds: chlortetracycline, demeclocycline, doxycycline, minocycline, oxytetracycline, tetracycline, and tigecycline (Garcia-Echauri et al., 2015). The emergence and spread of *tet(X)* has hindered the treatment of bacterial diseases. Class D β -lactamase that encodes an

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amoxicillin resistance gene *bla*_{OXA-347} have been reported to cause changes in sensitivity to cefotaxime, imipenem, and penicillin (Zangenah et al., 2017). This gene has been found in the gut microbiomes of humans and wild and domestic animals, as well as in raw wastewater (Cheng et al., 2012; Bougnom et al., 2020; Loo et al., 2020).

Since 2012, there have been few reports on plasmids in *R. anatipestifer* (Chen et al., 2012; Li et al., 2022). In this study, we found a plasmid in *R. anatipestifer* isolate RCAD0416 from Sichuan, China. This plasmid mediates multidrug resistance and can spread among different bacteria. The emergence of multidrug-resistance plasmids enables the spread of resistance genes.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, Primers and Culture Conditions

R. anatipestifer RCAD0416 was isolated from the respiratory tract of a healthy adult duck in Mianyang, Sichuan Province, China in 2017. Details of the bacterial strains and plasmids used in this study are listed in Table 1.

R. anatipestifer was cultured on tryptic soy agar (Oxoid Ltd., Basingstoke, UK) or in tryptic soy broth (Oxoid Ltd.) at 37°C. *E. coli* was cultured on Lysogeny Ager plates or Mueller–Hinton broth at 37°C. The following antibiotics were added, as required: 10 µg/mL florfenicol (FFC), 40 µg/mL kanamycin (KAN) or 100 µg/mL ampicillin (AMP).

Antibiotics Susceptibility Test

The following antibiotics were used in bacterial sensitivity tests: pipemidic acid (PIP), florfenicol (FFC), colistin (COL), azithromycin (AZI), erythromycin (ERM), norfloxacin (NOR), enrofloxacin (ENR), streptomycin (S); chloramphenicol (C), gentamicin (CN), lincomycin (MY), tetracycline (TET), tigecycline (TGC), doxycycline (DOX), ampicillin (AMP), cefoxitin (FOX), cephadrine (CH), oxytetracycline (OTC), minocycline (MIN), chlortetracycline (CTC), demeclocycline (DMC), cephalothin (CE), amoxicillin (AMO), imipenem (IPM), and cefepime (FEP). The above mentioned antibiotics were stored at a concentration of 10,240 µg/mL. All antibiotics were obtained from Dalian Meilun Biotech Co., Ltd. (Dalian, China).

Minimum inhibitory concentrations (MICs) were determined according to the performance standard of the Clinical and Laboratory Standards Institute (CLSI) (VET01, 2018). Hundred microlitre of bacterial solution and 100 µL of antibiotics of different concentrations were added to the 96-well microtiter plate, the final concentrations of the antibiotics ranged from 0.25 to 512 µg/mL. The *R. anatipestifer* isolate was diluted during the logarithmic growth period with TSB to OD₆₀₀ = 0.04, and the concentration of the bacterial inoculum was approximately 10⁶ CFU/mL. The inoculated microplates were incubated at 37°C for 24 h (Zhu et al., 2018). All studies were carried out in triplicate.

Whole-Genome Sequencing and Analysis

To obtain genetic information for RCAD0416, high-quality genomic and plasmid DNA were extracted with a TIANamp Bacteria DNA Kit (Tiangen Biotech Co, Ltd.,

Table 1. The strains and plasmids used in this study.

Strain or plasmid	Descriptions	Resource or references
Strains	Type strain	ATCC
<i>R. anatipestifer</i> ATCC 11845		
<i>R. anatipestifer</i> RCAD0416	Wild strain	Isolated from healthy adult duck. Sichuan, China
<i>E. coli</i> DH5α	Cloning host cell	Laboratory collection
<i>E. coli</i> DH5α	DH5α carrying pET28a-D1J36_09695	This study
(pET28a-D1J36_09695)	DH5α carrying pET28a-D1J36_09740	This study
<i>E. coli</i> DH5α	DH5α carrying pET28a-D1J36_09870	This study
(pET28a-D1J36_09740)	DH5α carrying pBAD24	This study
<i>E. coli</i> DH5α	DH5α carrying pBAD24- <i>bla</i> _{OXA-347}	This study
(pET28a-D1J36_09870)		
<i>E. coli</i> DH5α		
(pBAD24)		
<i>E. coli</i> DH5α		
(pBAD24- <i>bla</i> _{OXA-347})		
<i>E. coli</i> K-12 × 7232	EndA1 hsdR17 (rK-mk+) glnV44 thi-1 recA1 gyrA relA1	Roland et al. (1999)
<i>E. coli</i> K-12 × 7232 (pRCAD0416RA-1)	Δ(lacZYA-argF)U1692pir deoR (Φ80dlac Δ(lacZ)M15)	Laboratory collection
<i>E. coli</i> BL21(DE3)	<i>E. coli</i> K-12 × 7232 carrying pRCAD0416RA-1, FFC ^R	Laboratory collection
<i>E. coli</i> BL21(DE3) (pET28a)	expressing host cell	This study
<i>E. coli</i> BL21(DE3)	<i>E. coli</i> BL21(DE3) carrying pET28a	This study
(pET28a- <i>bla</i> _{OXA-347})	<i>E. coli</i> BL21(DE3) carrying pET28a- <i>bla</i> _{OXA-347}	
Plasmids		
pET28a	<i>E. coli</i> protein expression plasmid, IPTG-inducible	Laboratory collection
pBAD24	promoter, Kan ^R	Guzman et al. (1995)
	<i>E. coli</i> expression vector containing the arabinose	
	pBAD promoter. AMP ^R	

Abbreviations: ATCC, American type culture collection; AMP, ampicillin; FFC, florfenicol; KAN, kanamycin.

Beijing, China). Genome sequencing of *R. anatipestifer* RCAD0416 was conducted using PromethION (Oxford Nanopore Technologies, ONT) and Illumina PE150. Canu v1.5 (Koren et al., 2017) was used to assemble the filtered subreads. The assembled results were polished twice by Racon v3.4.3 (<https://github.com/isovic/racon>) (Vaser et al., 2017) using long reads. The output was further polished once by Pilon (v1.24, parameters, “—fix all”) (Walker et al., 2014) with the $100 \times$ Illumina reads. Final assemblies were trimmed, oriented and circularized using Circlator (v1.5.5, default parameters, <https://sanger-pathogens.github.io/circlator/>) (Hunt et al., 2015). Gene prediction was performed using Prodigal v2.6.3 (Hyatt et al., 2010) and NCBI Prokaryotic Genome Annotation Pipeline (PGAP, https://www.ncbi.nlm.nih.gov/genome/annotation_prok/) (Li et al., 2021). The genome completeness of the strain was evaluated using the CheckM14 genome quality estimator (Parks et al., 2015). Acquired antimicrobial resistance genes were predicted using the Comprehensive Antibiotic Resistance Database (Alcock et al., 2020)(CARD) and ResFinder 4.1 (Zankari et al., 2017; Bortolaia et al., 2020) (<https://cge.cbs.dtu.dk/services/ResFinder/>). The whole-genome sequence of *R. anatipestifer* RCAD0416 and plasmid sequence of pRCAD0416RA-1 have been deposited in GenBank under the accession number CP073239.1 and CP073240.1, respectively.

Transformation of pRCAD0416RA-1

To explore the resistance level mediated by the resistance gene for pRCAD0416RA-1, we transferred pRCAD0416RA-1 into *E. coli* K-12 \times 7232 via electroporation. The DH5 α bacterial solution was inoculated into LB broth and cultured to $OD_{600} = 0.8$. To prepare competent cells, the culture was centrifuged at 4000 r/min at 4°C for 10 min, use 30 mL chilled ddH₂O to resuspend cells, repeat the centrifugation and resuspension steps 2 times. Use 5 mL chilled 10% glycerol resuspend cells, centrifugal, 2 to 3 mL chilled 10% glycerol to resuspend again, dispense 100 μ L of each tube into EP tubes. Store at -80°C . Then, pRCAD0416RA-1 was extracted by TIANprep Mini Plasmid Kit (Tiangen Biotech Co, Ltd., Beijing, China) and used nanodrop to measure the concentration. 0.3 μ g, 0.5 μ g, and 1 μ g of plasmid were added to the competent cells. Electric shock conversion was performed under an electric field strength of 2.5 kV/cm. A volume of 200 μ L of LB was rapidly added to the cells. The cells were incubated at 37°C for 30 min. Transformants were selected using LB agar plates containing florfenicol (10 μ g/mL) (Zhao et al., 2018). The transformation efficiency was reported as the number of transformants/ μ g of DNA (Papagianni et al., 2007). Each experiment was repeated thrice.

Expression of bla_{OXA-347} and tet(X) Genes in E. coli

To confirm the function of bla_{OXA-347} in β -lactam resistance, bla_{OXA-347} was amplified with the primers

pBAD24-bla_{OXA-347}-F (5'-GGAATTCgttgtgcgcatctttgtgcgacag-3') and pBAD24-bla_{OXA-347}-R (5'-CCCAA GCTTggcacgaaagccggcaattatcggc-3'). The amplicon was linked to pBAD24 by restriction enzyme ligation. Three tet(X) genes in pRCAD0416RA-1 were amplified using primers with EcoRI and HindIII by PCR. The genes were then cloned into plasmid pBAD24 to produce pBAD24-tet(X). The 4 new plasmids were transferred to DH5 α and culture on Luria–Bertani agar (LB) medium containing 100 μ g/mL ampicillin. The transformants were verified by PCR and subjected to susceptibility tests using the broth microdilution method (Sun et al., 2020).

Protein tet(X) Structure Homology Modeling Using SwissModel

The amino acid sequences of the three tet(X) proteins on the plasmid were uploaded to SwissModel (Waterhouse et al., 2018).

Overexpression of the bla_{OXA-347} gene in E. coli BL21

We used the plasmid ligation method described above to construct pET28a-bla_{OXA-347}. Then, we transferred pET28a-bla_{OXA-347} to the *E. coli* BL21 strain. The successful transformants were grown overnight at 37°C in LB containing 50 μ g/mL kanamycin under continuous shaking (Dey et al., 2021). The cells were diluted with fresh LB (1:100) and cultured to $OD_{600} = 0.6$, after which 0.5 mM/L isopropyl β -D-1-thiogalactopyranoside (IPTG) was added. The cells were incubated for 4 h at 30°C under shaking at 180 r/m.

RESULTS

A Resistance Plasmid, pRCAD0416RA-1, Carrying the tet(X), ereD, ermF, AadS, bla_{OXA-347}, and floR Genes, was Found in R. anatipestifer

A multidrug-resistant RCAD0416 was isolated from ducks from Sichuan, China. The RCAD0416 strain was resistant to β -lactams, aminoglycosides, tetracyclines, macrolides, and florfenicol (Table 2). We used bacterial genome third-generation sequencing technology and a bioinformatics platform to construct and analyze the RCAD0416 genome completion map. We identified a plasmid in RCAD0416. We named the plasmid pRCAD0416RA-1. This circular plasmid consists of 37,356 nucleotides, has a GC content of 36.47%, and encodes 36 genes. The length of the gene coding region was 30,109 bp (80.6%). Seven of the predicted ORFs were hypothetical proteins; the other ORFs were assigned functions; where 8 ORFs were resistance genes. The genetic prediction results showed that pRCAD0416RA-1 is a resistance (R) plasmid. The genotype of the resistance gene was consistent with the antibiotic sensitivity

Table 2. MICs values of antibiotics tested in this study.

Strains	MIC (mg/L)																												
	Pipemidic Acids			Chloramphenicol		Colistin		Macrolides			Aminoglycosides					Lincomycins					Tetracyclines					β-lactams			
PIP	C	FFC	COL	CLA	ERM	NOR	ENR	S	CN	MY	MY	TET	TGC	OTC	MIN	CTC	DOX	DMC	AMP	FOX	CE	AMO	IPM	CEP					
RCAD0416	32	32	>1024	512	64	32	32	256	256	512	512	32	16						128	0.5				1					
7232	8	8	4	4	128	64	<0.25	8	2	512	512	8	1						4	16				64					
7232-pRCA-D0416RA-1	32	64	4	4	256	128	<0.25	8	1	512	512	32	8						32	128				128					
DH5α-pBAD24-tetX1												4	0.5	32	4	16	8	2											
DH5α-pBAD24-tetX2												8	4	64	4	32	8	4											
DH5α-pBAD24-tetX3												8	4	32	4	32	8	4											
BL21-pET28a												8	1	32	4	32	8	4											
BL21-pET28a- <i>bla</i> _{OXA-347}												4	4	32	4	32	8	4											
DH5α-pBAD24- <i>bla</i> _{OXA-347}												8	1	32	4	32	8	4											
DH5α-pBAD24- <i>bla</i> _{OXA-347}												8	1	32	4	32	8	4											
												4	4	32	4	32	8	4											
												256	32	8	2	2	2	2											
												>512	4	4	4	4	4	4											
												>512	32	8	4	4	4	4											

Abbreviations: AMP, Ampicillin; AMO, Amoxicillin; AZI, Azithromycin; C, Chloramphenicol; CN, Gentamicin; COL, Colistin; CTC, Chlorotetracycline; CH, Cephalothin; DMC, Demeclocycline; DOX, Doxycycline; ENR, Enrofloxacin; ERM, Erythromycin; FEP, Cefepime; FFC, Florfenicol; FOX, Cefoxitin; MY, Minocycline; NOR, Norfloxacin; PIP, Pipemidic Acid; S, Streptomycin; OTC, Oxytetracycline; TET, Tetracycline; TGC, Tigecycline.

(Figure 1). To determine the transferability of pRCA-D0416RA-1, we used electroporation to transfer pRCA-D0416RA-1 to *E. coli* K-12 × 7232. The transformation efficiency of electroporation was 8.82×10^2 CFU/μg DNA. The R plasmid caused a maximum 8-fold increase in the antibiotic sensitivity of the recipient. The most significant sensitivity changes were observed for β-lactams, florfenicol, chloramphenicol and tetracyclines. There was a 4- to 8-fold increase in the MIC of these antibiotics.

AMR Genes *tet(X)*, *bla*_{OXA-347}, and *florR* Mediate *R. anatipestifer* Resistance to Tigecycline, β-lactams, Florfenicol and Chloramphenicol

To compare the activity of the 3 plasmid-carrying *tet(X)* (D1J36_09695, D1J36_09740 and D1J36_09870), we constructed the 3 plasmids and conducted a susceptibility analysis. At the genetic level, the similarity between D1J36_09695 and D1J36_09740 was very high, reaching 99.57%. The amino acid sequence similarity for these 2 genes reached 98.97%. D1J36_09870 was quite different from the other 2 genes. In particular, 10 amino acids from the sequence of the other 2 proteins are deleted in the D1J36_09870 sequence. Existing reports on *tet(X)* show that changes or deletions in front of the amino acids encoded by *tet(X)* will create new *tet(X)* variants (Sun et al., 2019). Therefore, we speculate that amino acid deletions or mutations in this region will cause changes in the function of the *tet(X)* gene. The susceptibility test results provided evidence for our conjecture. The gene functions showed that the D1J36_09695 and D1J36_09740 have the same ability to mediate bacterial resistance to tetracycline drugs. D1J36_09695 and D1J36_09740 induce a higher level of resistance in bacteria than D1J36_09870. D1J36_09695 has 93.70% sequence identity with tetracycline destructase *tet(X)7*. D1J36_09740 and D1J36_09870 have 91.80% and 87.57% sequence identity, respectively, with the *tet(X)2* protein. The protein structure model (Figure 2) further supports that the 3 proteins belong to the *tet(X)* family. We also found that coexistence of the 3 genes in pRCAD0416RA-1 did not increase bacterial resistance to tetracycline. Multiple copies of the *tet(X)* gene are common in *R. anatipestifer* (Umar et al., 2021). This high carrying rate motivated us to explore the gene source. Most of the hits obtained for *tet(X)* using BLAST on NCBI were *Flavobacteriales*, *Weeksellaceae*, and *Riemerella*. Studies have identified *Flavobacteriaceae* (Zhang et al., 2020) or *Riemerella* as potential ancestral sources of the tigecycline resistance gene *tet(X)*. The BLAST results provided evidence for these inferences.

The gene *bla*_{OXA-347} is a class D beta-lactamase. In previous reports, *bla*_{OXA-347} was found to confer amoxicillin and meropenem resistance to bacteria (Zangenah et al., 2017). However, we did not find expression of this resistance gene in subsequent experiments. We found

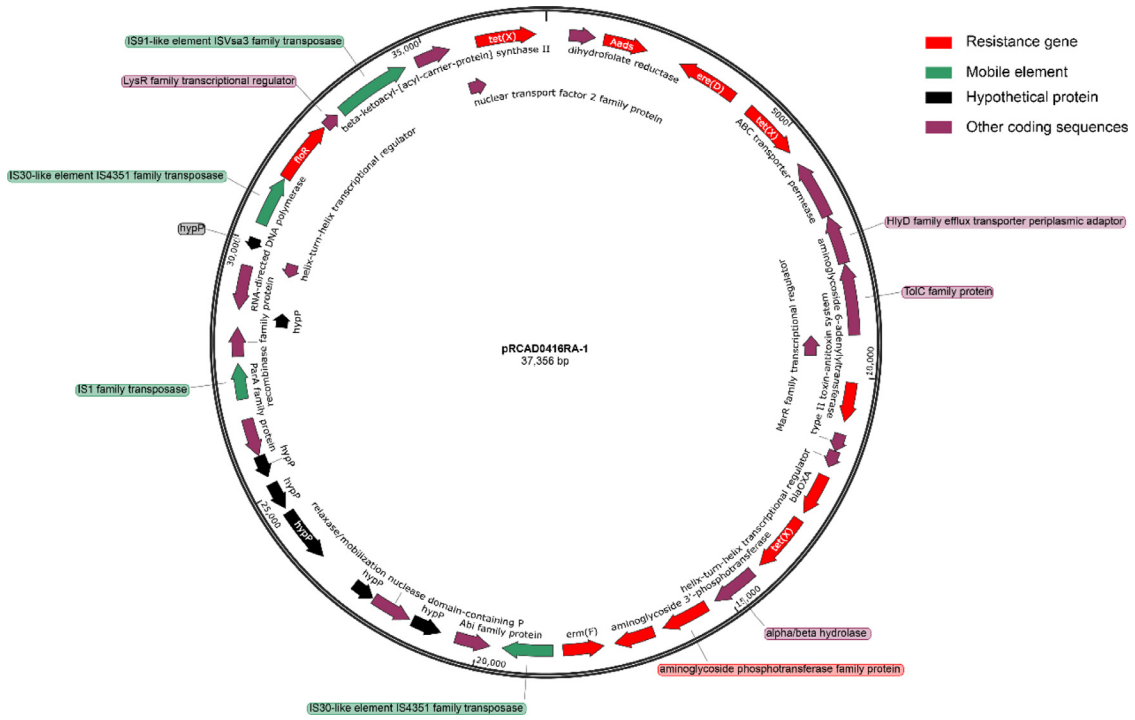


Figure 1. The plasmid profile of pRCAD0416RA-1 from *R. anatispestifer* strain RCAD0416. Genes with different functions are marked with different colors: red, antimicrobial resistance gene; green, mobile element; black, hypothetical protein; purple, other function.

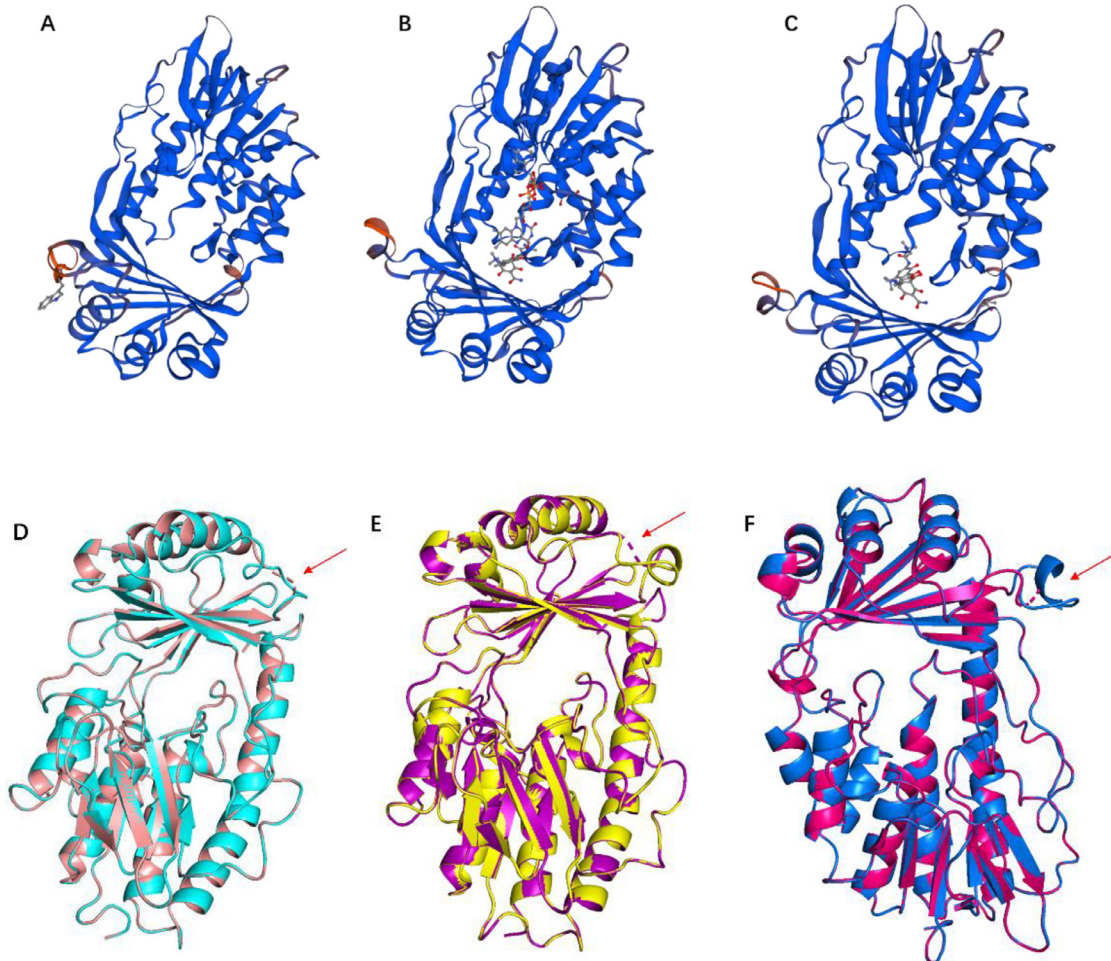


Figure 2. Structural modeling of (A) D1J36_09695, (B) D1J36_09740 and (C) D1J36_09870; cartoon representations of models of (D) *tet(X2)* (salmon) and D1J36_09695 (cyan), (E) *tet(X2)* (hot pink) and D1J36_09740 (yellow), and (F) *tet(X2)* (purple), and D1J36_09870 (deep blue). The arrow indicates a difference between models.

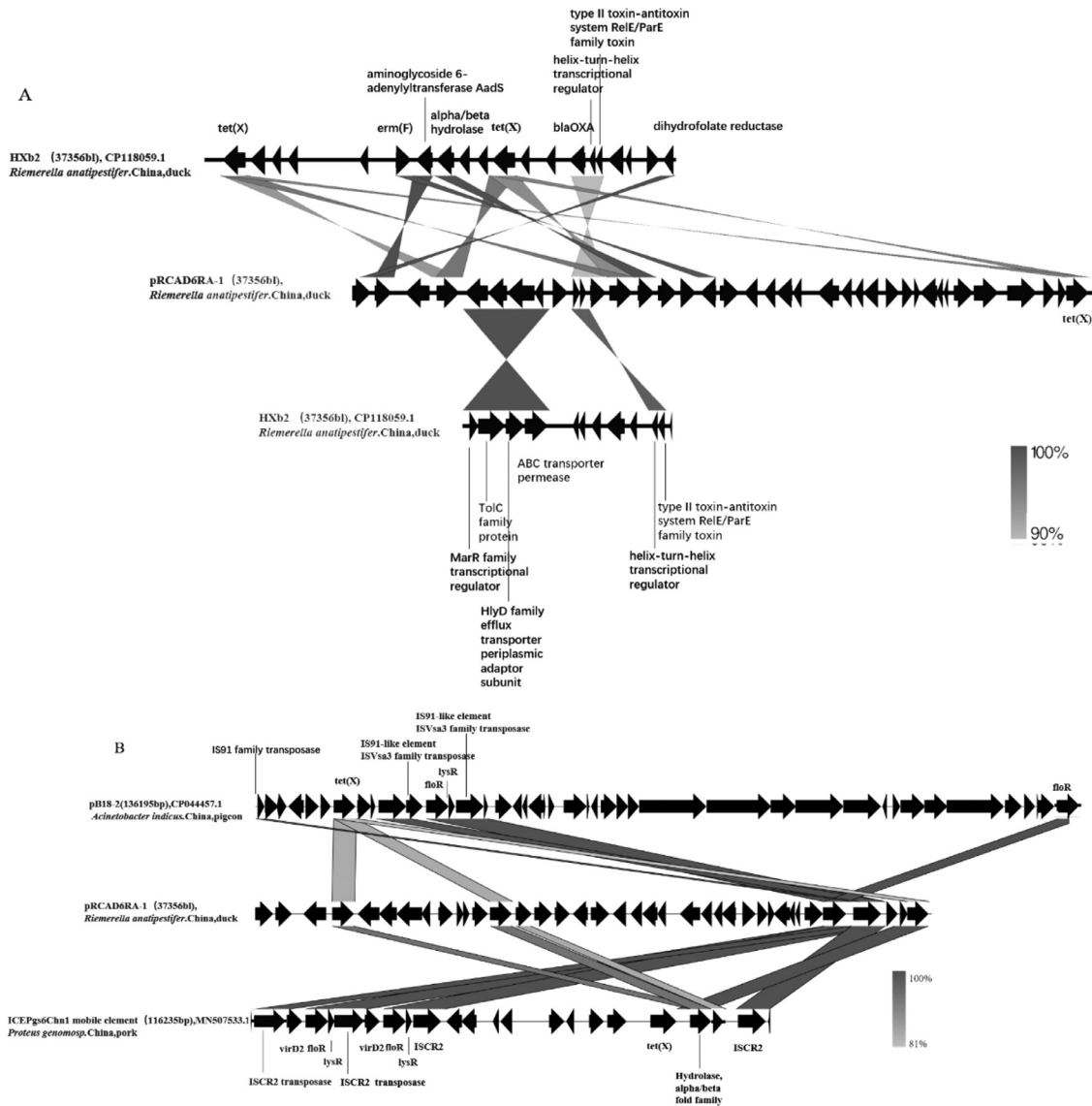


Figure 3. (A) Comparison of genomic fragment sequence of plasmid pRCAD0416RA-1 from *R. anatipestifer* strain RCAD0416 with the genome sequence of *R. anatipestifer* HXb2 (CP118059.1) (>90%) in the GenBank database, indicated by grey shading. (B) Comparison of genomic fragment sequence of plasmid pRCAD0416RA-1 from *R. anatipestifer* strain RCAD0416 with the sequence of plasmid pB18-2 (CP044457.1) and ICEPg6Chn1 mobile element (MN507533.1) retrieved from GenBank, indicated by gray shading. The grey shadow indicates a genetic similarity above 81%.

that this gene, especially after overexpression, mediates resistance to other β -lactam antibiotics, such as ampicillin, cephalothin, and cefoxitin (Table 1). *bla*_{OXA-347} can be transmitted between bacteria through transposons (Zangenah et al., 2017). Analysis of the upstream and downstream gene functions of *bla*_{OXA-347} revealed the IS30-like element IS4351 family transposase (Figure 1). This transposition pattern is also present in human gut flora (Cheng et al., 2012).

RCAD0416 is resistant to florfenicol and chloramphenicol. The MIC of florfenicol in transformants with pRCAD0416RA-1 increased 8-fold (from 8 mg/L to 64 mg/L), and the MIC of chloramphenicol increased 4-fold (from 8 mg/L to 32 mg/L). These results indicate that the resistance of the RCAD0416 strain to florfenicol may derive from this plasmid.

Fragments on Plasmids From Genome and Plasmids

The BLAST results from NCBI showed that the plasmid with the highest similarity was *R. anatipestifer* HXb2, with plasmid sequence coverage reaching 39%. Figure 3A shows that most of the resistance genes, such as *ermF*, *tet(X)*, *bla*_{OXA-347}, and *AadS*, were derived from the genome.

We also observed two matches, the *Acinetobacter indicus* strain B18 plasmid pB18-2 (18% coverage) and *Proteus genomosp.* 6 strain T60 ICEPg6Chn1 mobile element (22% coverage) (Figure 3B). The coverage of other matches was lower than 9%. The mobile elements on this plasmid are also the same as the above 2 plasmids.

DISCUSSION

The first plasmid in *R. anatipestifer* reported on NCBI (<https://www.ncbi.nlm.nih.gov/>) was found in Taiwan (Chang et al., 1998). We used the third-generation sequencing method to discover the plasmid from an *R. anatipestifer* strain in mainland China. Most of the fragments, especially the resistance genes, on the plasmid are from the HXb2 genome. However, there are no mobile elements related to transposition in these fragments. Mobile elements on this plasmid are from other plasmids and ICE. As the sources of the resistance genes and mobile elements are very different, it can be speculated that pRCAD0416RA-1 has recombined at least twice. Existing reports have demonstrated resistance genes of *R. anatipestifer* resulting from natural conversion. The emergence of multidrug-resistance plasmids is a means of disseminating resistance genes.

We tried to transfer the plasmid and found that its transfer ability was poor. We tried unsuccessfully to transfer the plasmid to ATCC 11845 and RA-CH-1 by natural transformation and to DH5 α by CaCl₂ transformation (data not shown). Only electroporation was successful for plasmid transfer. The conversion efficiency was low. As we mentioned earlier, the full length of pRCAD0416RA-1 is 37356 bp, which may affect plasmid transformability. Electroporation creates holes in a cell membrane through transient currents, allowing DNA from foreign macromolecules to enter the cell. Apart from the current study, few reports on plasmids in *R. anatipestifer* have appeared since 2012. The scarcity of plasmids may explain their poor transformability. According to the results of the plasmid sequence alignment, the plasmid may have undergone recombination. Mobile originals on plasmids provide opportunities for recombination. Under the influence of the transposon, *floR* can transfer this (Walker et al., 2014) plasmid from other bacilli. We found that concomitant existence of *tet(X)* in the sequenced *R. anatipestifer* genome with *bla*_{OXA} (Li et al., 2022). This type of adjoining mode also exists in pRCAD0416RA-1.

We transferred the plasmid to *E. coli* K-12 \times 7232 and found changes in the antibiotic sensitivity to β -lactams, tetracyclines, florfenicol, and chloramphenicol. An analysis of the sequencing results showed that the plasmid carries resistance genes for β -lactams, florfenicol, aminoglycosides, tetracyclines, and erythromycin. Class D β -lactamase *bla*_{OXA-347} has been found in the gut microbiomes of humans and wild and domestic animals, as well as in raw wastewater (Cheng et al., 2012; Bougnom et al., 2020; Loo et al., 2020). This type of β -lactamase confers carbapenem resistance to bacteria. The resistance gene *bla*_{OXA-347} has been reported to mediate bacterial resistance to amoxicillin. However, in this study, we did not find that *bla*_{OXA-347} conferred carbapenem and amoxicillin resistance to *R. anatipestifer* and *E. coli*. A gene function analysis showed that only over-expression of *bla*_{OXA-347} resulted in an increased level of resistance. We have found in previous studies that strains (*Capnocytophaga cynodegmi* and *Capnocytophaga*

stomatis) carrying this gene are resistant to cefotaxime, imipenem, penicillin G, and amoxicillin. However, the resistance phenotype cannot represent the resistance level of *bla*_{OXA-347} because no single gene function has been verified. The resistance phenotype may also be affected by other factors, such as the existence of a non-specific efflux mechanism and unknown resistance genes or resistance mechanisms. The *bla*_{OXA-347} protein has 88.85% identity and 98.35% coverage with *bla*_{OXA-347} in the strains mentioned above. Moreover, pRCAD0416RA-1 is a low-copy-number plasmid vector. The copy number of a plasmid affects the resistance level. An increase in the plasmid copy number has been found to contribute to elevated carbapenem resistance in *K. pneumoniae* carrying *bla*_{OXA} (Shen et al., 2020). This result may explain the low resistance to β -lactam antibiotics.

There are three *tet(X)* genes in pRCAD0416RA-1. Two of these genes mediates bacterial resistance to tige-cycline, and the resistance level is low (8-fold). These genes have been found to mediate low-level tige-cycline resistance. This result was confirmed in a study on *tet(X14)* (identity and coverage >99%) (Cheng et al., 2020). The third gene does not have a clear resistance phenotype but can also change the sensitivity of *E. coli* to tetracycline. Multiple copies of *tet(X)* commonly exist in *R. anatipestifer* (Umar et al., 2021; Li et al., 2022). This phenomenon has also been reported in Gram-positive bacteria (Li et al., 2020). We are thus led to consider the significance of this coexistence relationship. A high-level structural analysis shows that all 3 proteins belong to the *tet(X)* family. However, there are differences among the structures and ligand binding sites of the 3 proteins. However, the resistance phenotypes of D1J36_09695 and D1J36_09740 are highly similar. The existence of multiple *tet(X)* has no superposition or synergistic effect on the resistance level. Moreover, when *R. anatipestifer* carries multiple *tet(X)*, the level of resistance is not necessarily higher than that mediated by a single *tet(X)*.

In summary, we report the discovery of wild plasmid pRCAD0416RA-1 in *R. anatipestifer*. This plasmid carries a gene that mediates resistance to β -lactams, erythromycin, florfenicol, chloramphenicol, tetracyclines, and aminoglycosides. We transformed the plasmid into other strains through experiments and verified the mobility of the plasmid. We explored the function of plasmid-mediated *tet(X)*, *floR*, and *bla*_{OXA-347} and analyzed the origin of genes. We concluded that the resistance genes on the plasmid were transferred from the genome or plasmid of other strains through recombination.

DISCLOSURES

The authors declare no competing interests.

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