Identification of floR Variants Associated With a Novel Tn4371-Like Integrative and Conjugative Element in Clinical Pseudomonas aeruginosa Isolates

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Florfenicol is widely used to control respiratory diseases and intestinal infections in food animals. However, there are increasing reports about florfenicol resistance of various clinical pathogens. floR is a key resistance gene that mediates resistance to florfenicol and could spread among different bacteria. Here, we investigated the prevalence of floR in 430 Pseudomonas aeruginosa isolates from human clinical samples and identified three types of floR genes (designated floRv, floR-T1 and floR-T2) in these isolates, with floR-T1 the most prevalent (5.3%, 23/430). FloR-T2 was a novel floR variant identified in this study, and exhibited less identity with other FloR proteins than FloRv. Moreover, floR-T1 and floR-T2 identified in P. aeruginosa strain TL1285 were functionally active and located on multi-drug resistance region of a novel incomplete Tn4371-like integrative and conjugative elements (ICE) in the chromosome. The expression of the two floR variants could be induced by florfenicol or chloramphenicol. These results indicated that the two floR variants played an essential role in the host’s resistance to amphenicol and the spreading of these floR variants might be related with the Tn4371 family ICE.

Keywords: floR, florfenicol resistance, Pseudomonas aeruginosa, Tn4371, integrative and conjugative elements
INTRODUCTION

Florfenicol is a fluorinated synthetic analog of thiamphenicol (Syriopoulou et al., 1981), mainly used to control respiratory tract diseases and enteric infections in food-producing animals (Zhao et al., 2016). However, due to inappropriate medication use, florfenicol resistance has become increasingly serious in veterinary medicine (Chang et al., 2014). Although florfenicol is not approved for use in humans, an increasing number of studies have reported dramatic and serious florfenicol resistance in various clinical strains, such as Pasteurella multocida, Salmonella, and Klebsiella pneumoniae (Lu et al., 2018; Ujvari et al., 2019; Zhan et al., 2019).

To date, seven florfenicol resistance genes (excluding variants), floR, fexA, fexB, opraA, opxA and estDL136, have been reported (Arcangiolli et al., 1999; Schwarz et al., 2000; Kehrenberg and Schwarz, 2004; Schwarz et al., 2004; Lang et al., 2010; Liu et al., 2012; Tao et al., 2012; Wang et al., 2015). Among them, floR is one of the main florfenicol resistance genes in Gram-negative bacteria (He et al., 2015). Several variants of the floR gene, including pp-flo, cmfA-like, flofV and flofSt, have been documented, and most of them encode 404 aa proteins. These floR variants are closely related to each other, and flofV from Stenotrophomonas maltophilia shares the lowest amino acid identity (88.4%-91.8%) with the others excluding pp-flo (He et al., 2015). The floR gene has been identified either on chromosomes or plasmids of various bacteria and has often been associated with mobile genetic elements and genomic islands (Lai et al., 2013; Gabida et al., 2015; da Silva et al., 2017).

Pseudomonas aeruginosa is an opportunistic pathogen that can cause numerous acute or chronic infections, and is notorious for its intrinsic and acquired resistance to numerous antibiotics (Breidenstein et al., 2011; Domalaon et al., 2018). Generally, P. aeruginosa chromosomes do not carry the floR gene. Although P. aeruginosa is clinically resistant to chloramphenicol (Morita et al., 2014), rifampicin-tobramycin conjugates could break the intrinsic resistance of P. aeruginosa to chloramphenicol in vitro and in vivo, making it suitable for clinical treatment (Idowu et al., 2019). However, the floR gene carried by this pathogen may cause this strategy to fail when chloramphenicol is used. The prevalence of the floR gene in P. aeruginosa hasn’t been previously investigated. In this study, we determined the prevalence of floR gene among 430 clinical P. aeruginosa isolates collected from Wenzhou, China in the years 2008-2009 and 2015-2017. The combination of whole-genome sequencing, genotyping and gene expression methods was used to characterize the floR variants. A novel Tn4371-like integrative and conjugative element (ICE) carrying floR-T1 and floR-T2 was identified, which indicated that the Tn4371-like ICE might play an important role in the dissemination of floR-T2.

MATERIALS AND METHODS

Bacterial Isolation

A total of 430 clinical P. aeruginosa strains isolated from sputum, urine or blood samples of patients were collected from a teaching hospital of Wenzhou Medical University. Among these isolates, 200 strains were isolated during 2008-2009, and 230 strains were isolated in 2015-2017. The strains were identified using the Vitek-60 microorganism auto-analysis system (BioMerieux Corporate, Craponne, France).

Antimicrobial Susceptibility Testing

Minimum inhibitory concentrations (MICs) of 17 antimicrobial agents were determined using an agar dilution method with Mueller-Hinton agar recommended by the Clinical and Laboratory Standards Institute (CLSI document M100-S7, 2017). Broad range concentrations of 0.125-1024 μg/mL were used for all the agents. MICs were interpreted according to CLSI breakpoints for P. aeruginosa.

DNA Extraction and Sequencing

Each purified isolate was incubated overnight in 5 ml of Luria-Bertani (LB) broth at 37°C for 16 hours, and genomic DNA was extracted using an AxyPrep Bacterial Genomic DNA Miniprep kit (Axygen Scientific, Union City, CA, USA). According to the time period of isolation, two mixed DNA collections consisting of equal amounts of genomic DNA of each strain were obtained. One collection (designated TL0809) contained the bacteria isolated from 2008-2009 and the other (designated TL151617) contained those isolated among 2015-2017. The library with an average insert size of 400 bp was prepared using NEBNext Ultra II DNA library preparation kit, and subsequently high-throughput sequenced by the Illumina Novaseq (paired-end run; 2×150 bp). In addition, a 10- to 20-kb insert library was obtained from the genomic DNA of P. aeruginosa TL1285 and sequenced by Pacific Biosciences RSII sequencers at Annoroad Gene Technology Co., Ltd. (Beijing, China).

Genome Assembly, Annotation, and Bioinformatics Analysis

Genome assembly of pooled DNA sequencing data was performed using megapath (Li et al., 2015), and contigs less than 400 bp were discarded. The complete genome of P. aeruginosa TL1285 was assembled using Canu (Koren et al., 2017) with long reads obtained from PacBio sequencing. Error correction of tentative complete circular sequence was performed using Pilon (Walker et al., 2014) with short read sets derived from Illumina sequencing. Open reading frames (ORFs) of pooled DNA sequences were predicted using Prodigal (Hyatt et al., 2010) with default parameters. Using the antibiotic resistance genes of the CARD (Jia et al., 2017) and ResFinder (Ea et al., 2010) with default parameters. Using the antibiotic resistance genes of the CARD (Jia et al., 2017) and ResFinder (Ea et al., 2010) databases as a query, a BLASTN search was performed against the two assembled sequences of the pooled DNA with thresholds of >70% nucleotide identity and >80% alignment coverage. Gene prediction and annotation of TL1285 were initially performed with RAST (Aziz et al., 2008) and then verified by BLASTP searches against the UniProtKB/Swiss-Prot (Boutet et al., 2016) and RefSeq (O’Leary et al., 2016) databases. Annotation of mobile genetic elements was carried out using online databases including ISfinder (Sigier et al., 2006), INTEGRALL (Moura et al., 2009), and the Tn Number Registry (Roberts et al., 2008). Comparison of the TL1285 genome with the other six genomes was performed using
BLAST Ring Image Generator (Alikhan et al., 2011). Gene organization diagrams were generated using R script and modified with Inkscape 1.0 (https://inkscape.org/en/).

**PCR Amplification and Cloning of the floR Gene**

Genomic DNA of each of the 430 isolates was screened for the floR gene using PCR with primers listed in Table 1. PCR amplification was carried out under the following conditions: initial denaturation for 10 min at 94°C, 35 cycles of denaturation (30 s at 94°C), annealing (30 s at 58°C) and extension (90 s at 72°C) and a final extension for 10 min at 72°C. The floR-T1 and floR-T2 gene sequences with promoter regions were amplified from *P. aeruginosa* TL1285 and cloned into pUCP24. Electroporation transformation was used to introduce the recombinant plasmids into *P. aeruginosa* PAO1 by Bio-rad MicroPulser with a voltage at 2.6 kv, resistance at 200 Ω and pulse time of 5 ms (Dennis and Sokol, 1995).

**Comparison of the Expression of floR-T1 and floR-T2**

Quantitative reverse transcription PCR (qRT-PCR) was used to investigate the expression of the floR variants of TL1285 and transformants in the presence or absence of 2 mg/L florfenicol or chloramphenicol. In brief, RNA was extracted from 3 mL of LB broth culture (OD600 = 1) of *P. aeruginosa* TL1285 and the transformants using TRizol Reagent (Invitrogen, USA) following the manufacturer’s instructions. RNA (1 μg) was used as the template for cDNA synthesis using HiScript II Reverse Transcriptase (Vazyme, Nanjing, China) following the manufacturer’s instructions. qRT-PCR was used to quantify the amount of floR-T1 and floR-T2 in cDNAs using ChamQ Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China) following the manufacturer’s instructions with the qPCR primers (Table 1).

**Detection of the Extrachromosomal Intermediate**

Inverse PCR using the primers beside the attL and attR sites could be utilized for the rapid identification of the extrachromosomal intermediate of Tn4371 (Ryan et al., 2009). PCR product was obtained only when integrative and conjugative element (ICE) was excised from the chromosome and circularized. Since no attL site was identified in TL1285, we designed two primers (P2 and P3) located beside the integrase genes as the forward primers. PCR amplification was carried out under the following conditions: an initial denaturation of 10 min at 94°C; 33 cycles of denaturation (94°C for 30 s), annealing (62°C for 30 s), and extension (72°C for 90 s); and a final extension step at 72°C for 10 min.

**GenBank Accession Number**

The complete chromosome sequence of the *P. aeruginosa* TL1285 (CP053390) has been submitted to NCBI GenBank.

**Ethics Approval**

This study uses strains obtained from a teaching hospital of Wenzhou Medical University. It did not require the study to be reviewed or approved by an ethics committee because individual patient data was not involved, and only anonymous clinical residual samples during routine hospital laboratory procedures were used in this study.

**RESULTS**

**Florfenicol and Chloramphenicol MICs of the Strains**

The MICs of florfenicol and chloramphenicol were determined for the 430 clinical *P. aeruginosa* isolates. It showed that 21

| TABLE 1 | PCR primers used in this study. |
|-----------------|-----------------|-----------------|
| **Primers** | **Purpose** | **Sequences** |
| s-flor-F | screening of floR | GCGCAACGGTGTCTGCATT |
| s-flor-R | | GCATTGCCAGTATAAGCCAAA |
| s-flor-T1-F | screening of floR-T1 | GCGCAACGGGTTCGTGCTT |
| s-flor-T1-R | | GCGGAAGCCAGTGCAGCAAGT |
| s-flor-T2-F | screening of floR-T2 | GGCCATACCTTTCTACGTC |
| s-flor-T2-R | | TCAAGCCGCAAGCAAGACG |
| c-flor-T1-F | cloning of floR-T1 | GGGATTGCGTGAGAAATGCTAG |
| c-flor-T1-R | | ATTGAAGGSGGTATCTTCCGACAG |
| c-flor-T2-F | cloning of floR-T2 | AACCTCATGATGGTCGCGTCC |
| c-flor-T2-R | | ATTTGAAACCCGCTTTCAAG |
| q-flor-T1-F | floR-T1 qRT-PCR | GCGGACGTATAGCGCAACTG |
| q-flor-T1-R | | TCTTGACATCGCTTTAAGAG |
| q-flor-T2-F | floR-T2 qRT-PCR | ATCTTGCGAGTGCCAGCATC |
| q-flor-T2-R | | TCTGGCGACAAAGGACTTC |
| PA165-F | *P. aeruginosa* 16S rRNA qRT-PCR | ACGCGGAAGGACCTTACCC |
| PA165-R | | AAAGGTTGCGCTGCTGATAC |
| EC165-F | E. coli 16S rRNA qRT-PCR | ATTGCCAAGGCGAATAGCG |
| EC165-R | | CTAGGTTACCTGTGTAAGA |
| Tn4371-P1 | circular forms and insertion sites of Tn4371 | CGAAGGCGGCTAAAGCTGACCT |
| Tn4371-P2 | | GAAGCTGCGAGGACGGTGGT |
| Tn4371-P3 | | CAAGGATCGGCGCTTGATGCT |
(4.88%) and 23 (5.35%) of the strains exhibited much higher resistance levels to florfenicol and chloramphenicol with the same MICs of ≥512 μg/mL for them (Figure 1). A total of 94.65% (407/430) of the strains were resistant to either florfenicol or chloramphenicol (or both), and only 5.35% (23/430) isolates were susceptible to both florfenicol and chloramphenicol with MIC ≤16 μg/mL.

**Identification of the floR Variants**

To investigate the prevalence of the floR gene among clinical P. aeruginosa isolates, two mixed DNA collections TL0809 (containing 200 P. aeruginosa strains isolated from 2008-2009) and TL151617 (containing 230 P. aeruginosa strains isolated from 2015-2017) were sequenced. Using the floR gene (AF231986) as a reference, three types of floR variants (the reference floR, floR-T1 and floR-T2 in this study) were identified in the two mixed genomes, of which TL0809 contained all three and TL151617 contained only two floR variants (the reference floR and floR-T1) (Table 2). Other antimicrobial resistance genes (ARGs) identified in TL0809 and TL151617 were listed in Table S1.

The result of PCR amplification of the reference floR, floR-T1 and floR-T2 genes showed that the most prevalent variant was floR-T1, while floR-T2 was only identified in the isolates collected from 2008-2009 (Table 2). The positive rates were consistent with the abundance [expressed as ‘copy of ARG per copy of 16S-rRNA gene’ (Li et al., 2015)] of the corresponding genes in the pooled genomic DNA sequencing libraries. The positive rate of the floR-T1 gene in the strains collected from 2015-2017 (7.39%, 17/230) was higher compared with that from 2008-2009 (3.00%, 6/200).

**Antimicrobial Susceptibility of P. aeruginosa TL1285 and the Recombinants With the Cloned floR Variants**

Among all P. aeruginosa strains, only one strain named TL1285, isolated from a sputum sample in 2008, carried both floR-T1 and floR-T2. P. aeruginosa TL1285 was resistant to chloramphenicol, florfenicol and many other antibacterial agents (Table 3). The fragment containing floR-T1 or floR-T2 gene and its putative promoter region was amplified from TL1285 genomic DNA and subsequently cloned into pUCP24, and then transformed into E. coli DH5α and P. aeruginosa ΔPAO1 (P. aeruginosa PAO1 deleted of ampG), respectively. As a result, compared with the recipients (E. coli DH5α and P. aeruginosa ΔPAO1), the recombinants with the cloned floR-T1 (DH5α/pUCP24-floR-T1 and ΔPAO1/pUCP24-floR-T1) increased ≥4 folds of MIC levels to both chloramphenicol and florfenicol and the recombinants with the cloned floR-T2 (DH5α/pUCP24-floR-T2 and ΔPAO1/pUCP24-floR-T2) increased ≥8 folds of MIC levels to both chloramphenicol and florfenicol.

**FIGURE 1** | MIC results of the 430 clinical P. aeruginosa isolates against florfenicol and chloramphenicol.

**TABLE 2 |** The abundance and PCR positive rates of the floR variants.

| floR variants | Identity | Match length (aa) | Abundance | PCR positive rate |
|---------------|----------|-------------------|-----------|------------------|
| TL0809        | floR<sup>a</sup> | 99.3% | 404        | 0.68             | 2/200 (1.00%) |
|               | floR-T1    | 91.3% | 404        | 1.18             | 6/200 (3.00%) |
|               | floR-T2    | 87.6% | 404        | 0.32             | 1/200 (0.50%) |
| TL151617      | floR       | 99.3% | 404        | 0.56             | 3/230 (1.30%) |
|               | floR-T1    | 91.3% | 404        | 4.95             | 17/220 (7.29%) |

<sup>a</sup>reference floR (AF231986).
chloramphenicol and florfenicol, respectively. The results indicated that the floR-T1 and floR-T2 genes of P. aeruginosa TL1285 were functionally active.

**Expression of the floR Variants**

The expression of the two floR variants with or without florfenicol (or chloramphenicol) induction were detected (Figure 2). It revealed that the mRNA levels of floR-T2 in P. aeruginosa TL1285 and the corresponding transformants (DH5α/pUCP24-floR-T2 and ΔPAO1/pUCP24-floR-T2) were significantly increased, while the mRNA levels of floR-T1 in P. aeruginosa TL1285 and the transformants (DH5α/pUCP24-floR-T1 and ΔPAO1/pUCP24-floR-T1) were only slightly increased in the presence of florfenicol or chloramphenicol.

**Characterization of the floR Variants**

Using phylogenetic analysis, the amino acid identities of FloR-T1 and FloR-T2 with the known FloR proteins ranged from 90.80% to 100% and 86.10% to 88.90%, respectively (Figure S1). FloR-T1 was identical to the FloR protein (YP_001715371.1) identified in Acinetobacter baumannii, while FloR-T2 showed the highest identity (88.90%) with the FloR protein (YP_005351917.1) identified in Klebsiella pneumoniae.

The translational attenuator that consisted of a single pair inverted repeat (IR) sequence, and a short reading frame of 6-9 aa peptide was identified upstream of the floR variants (Figure 3). IR1 and IR2 can form a stable stem-loop structure blocking the resistance gene-associated ribosome binding site (RBS). The short peptides of floR-T1 and floR-T2 differ in three amino acids. The attenuator sequences of floR-T2 and floRv encode an identical peptide, although one synonymous variation (A>T) in their nucleotide sequences. The attenuators’ IR resulted also differently, and the stem-loop structures formed in distinct stable states. Among these variants, floR-T2 and floRv showed the most stable structure. However, the stable stem-loop structure of the attenuator sequence did not overlap with the RBS site of the floR gene.

| Antibiotics | TL1285 | DH5α | DH5α/pUCP24-floR-T1 | DH5α/pUCP24-floR-T2 | ΔPAO1 | ΔPAO1/pUCP24-floR-T1 | ΔPAO1/pUCP24-floR-T2 |
|-------------|--------|------|----------------------|----------------------|-------|----------------------|----------------------|
| Ampicillin  | 1024   | –    | –                    | –                    | –     | –                    | –                    |
| Ceftazidime | <1     | –    | –                    | –                    | –     | –                    | –                    |
| Levofloxacin| <0.5   | –    | –                    | –                    | –     | –                    | –                    |
| Cefpyridine | 4      | –    | –                    | –                    | –     | –                    | –                    |
| Minocycline | 64     | –    | –                    | –                    | –     | –                    | –                    |
| Chloramphenicol | 128      | 4    | 64                   | 64                   | 32    | 128                  | 512                  |
| Florfenicol | 256    | 4    | 64                   | 128                  | 32    | 256                  | >1024                |
| Ciprofloxacin | 2     | –    | –                    | –                    | –     | –                    | –                    |
| Azithromycin | 32     | –    | –                    | –                    | –     | –                    | –                    |
| Fosfomycin  | 256    | –    | –                    | –                    | –     | –                    | –                    |
| Tigecycline | 4      | –    | –                    | –                    | –     | –                    | –                    |
| Colistin    | <1     | –    | –                    | –                    | –     | –                    | –                    |
| Erythromycin| 256    | –    | –                    | –                    | –     | –                    | –                    |
| Nalidixic acid | >1024     | –    | –                    | –                    | –     | –                    | –                    |
| Gentamicin  | >1024  | –    | –                    | –                    | –     | –                    | –                    |
| Kanamycin   | 64     | –    | –                    | –                    | –     | –                    | –                    |
| Streptomycin| >1024  | –    | –                    | –                    | –     | –                    | –                    |

**TABLE 3** | MIC results of P. aeruginosa TL1285 and recombinants to 17 antibiotics (µg/mL).
Whole genome sequencing (WGS) was performed for *P. aeruginosa* TL1285 carrying both floR-T1 and floR-T2, and only produced a circular 6,609,407 bp chromosome with an average GC content of 66.06% encoding 5,611 ORFs. Multiple ARGs, including resistance genes for β-lactams (*bla*OXA-50 and *bla*PDC-3), aminoglycosides (*aadA5* and *aac(3)-IIa*), sulfonamides (*sul1*), tetracycline (*tetG*), chloramphenicol (*catB7*, *floR-T1* and *floR-T2*) and fosfomycin (*fosA*), were identified in the *P. aeruginosa* TL1285 genome. The flofuscin-resistant genes *floR-T1* and *floR-T2* were embedded in an 86-kb Tn4371-like integrative and conjugative element (ICE) (Figure 4).

To track the epidemiological correlation between floR-T2 and genome islands, a BLASTN search was performed against the GenBank database using floR-T2 as a query. A total of five *P. aeruginosa* chromosomes, WPB099 (CP031878), WPB100 (CP031877), WPB101 (CP031876), PASGNDM345 (CP020703) and PASGNDM699 (CP020704), and one *E. cloacae* chromosome, AR_038 (CP030347), were found carrying floR-T2. Through MLST analysis, the five *P. aeruginosa* belonged to ST308, while TL1285 to ST316. Interestingly, these floR-T2-carrying strains came from different sources. WPB099, WPB100 and WPB101 were isolated from hospital wastewaters in Singapore, PASGNDM345 and PASGNDM699 from patient sputum in Singapore, while *E. cloacae* AR_038 and TL1285 were from patient sputum collected in United States and China, respectively. Whole genome alignment of the six *P. aeruginosa* strains revealed high identity, and their differences were mainly in some genomic islands (Figure 4). The Tn4371-like ICE carrying floR-T2 in TL1285 was also partially present in these five *P. aeruginosa* strains. Nevertheless, it should be noted that WPB099, WPB100 and WPB101 were not fully sequenced, and the floR-T2 gene was located on an approximately 10 kb separate segment, which means the precise genetic environments around floR-T2 could not be described.

Comparative analysis of the Tn4371-like ICE regions of six *P. aeruginosa* strains revealed that the plasmid maintenance system (*repA, parA* and *parB*) and conjugational transfer systems were conserved (Figure 5). The variable region between the *traF* and *traR* genes, which encoded a biphenyl catabolic *bph* gene cluster in Tn4371 (AJ536756), was different in these six *P. aeruginosa* isolates. The variable regions of WPB099, WPB100 and WPB101 were a 20-kb fragment encoding the *oqxB32* gene, which confers resistance to quinolone. The variable regions of PASGNDM345 and PASGNDM699 shared high identity with those of WPB099, WPB100 and WPB101. The only difference was that in PASGNDM345 and PASGNDM699, a 13.7-kb fragment flanked by 695 bp direct repeats was inserted between *czcD* and *lysR*, which encode *bla*NDM-1, *msr(E)* and floR-T2 genes. The variable region of TL1285 was similar to those of PASGNDM345 and PASGNDM699, except that the *bla*NDM-1-*hp-msr(E)* genes of PASGNDM345 and PASGNDM699 were replaced by floR-T1-tetR-tetA-lysR in TL1285.

The integrase genes (*int*) of PASGNDM345, PASGNDM699, WPB99, WPB100 and WPB101 were identical and shared 78% identity with that of Tn4371. However, no homologue of
intTn4371 was found in TL1285 (Figure 5). Tn4371 family ICEs could be integrated into the genome through an 8-bp attB site, generating direct repeat attL and attR element chromosomal junctions (Merlin et al., 1999). In PASGNDM345, PASGNDM699, WPB99, WPB100 and WPB101, 8-bp repeats (5’-TTTTTTGT-3’) were identified in both extremities of the ICE region. However, in TL1285, only attR was found (Figure 5). The noc gene upstream of repA in TL1285 was truncated by a novel Tn402 family transposon. The transposon is formed by ISCfr1 and In2 carrying a single aadA5 cassette embedded

FIGURE 4 | Sequence conservation among P. aeruginosa TL1285 and 6 other genomes carrying floR-T2. From innermost to outermost: Circle 1 shows the scale in kb; Circles 2 and 3 represent the GC content and GC skew maps of TL1285, respectively; Circle 4 represents the genome of TL1285; Circles 5-10 represent the homologous regions of PASGNDM345, PASGNDM699, WPB099, WPB100, WPB101 and AR_038 compared to those of TL1285, while the regions without similar hits between them were left blank; Circle 11 displays the genomic islands in TL1285; Circle 12 displays the antibiotic resistance genes in TL1285.

FIGURE 5 | Comparative genomic analysis of the ICE region of TL1285 and 5 other floR-T2-carrying P. aeruginosa isolates. Genes with different functions are shown in different colors: red, transposable elements; yellow, drug resistance; orange, conjugational transfer; blue, plasmid maintenance; purple, replication; brown, genes with other functions; white, hypothetical proteins.
downstream of the *tnpR* gene of *Tn1013*, and this *Tn402* family transposon was surrounded by 37-bp imperfect inverted repeats (Figure S2).

Inverse PCR using primers P1, P2 and P3 (Figure 5) was performed to detect whether the ICE in TL1285 could generate a circular extrachromosomal form, but no positive result was observed. Taken together, we speculate that the ICE in TL1285 is an incomplete member of the *Tn4371* family and may have lost the exciting or integrating ability. The insertion of the *Tn402* family transposon leads to the loss of the upstream sequence of the nucleoid occlusion protein coding gene *noc*, including the integrase gene *int* of ICE.

**DISCUSSION**

In this work, we found that among the 430 clinical *P. aeruginosa* isolates detected, most (94.65%, 407/430) of them were resistant to florfenicol or/and chloramphenicol. In fact, *P. aeruginosa* was intrinsically resistant to amphenicols, and the MICs to florfenicol and chloramphenicol for *P. aeruginosa* ATCC27853 were both 64 µg/mL (Fass and Barnishan, 1979). Active efflux and chloramphenicol acetyltransferase (CAT) encoded on *P. aeruginosa* chromosome are two major mechanisms of amphenicol resistance (Nitzan and Rushansky, 1981; Li et al., 1994), and different types of CAT determinant also contribute to amphenicol resistance (White et al., 1999). However, there were still 5.35% (23/430) isolates susceptible to amphenicol. The *amphenicol* resistance (White et al., 1999). However, there were still 5.35% (23/430) isolates susceptible to amphenicol. The mutation and deletion of multidrug efflux system (such as MexA-MexB-OprK) and other resistance mechanisms might play a role in the loss of resistance to florfenicol or/and chloramphenicol of these bacteria.

Three *floR* variants (*floR, floR-T1* and *floR-T2*) were identified in a number of clinical *P. aeruginosa* isolates, in which *floR-T1* was the most prevalent variant and *floR-T2* was a novel variant identified in this study. The positive rate of the *floR-T1* gene in the strains collected from 2015-2017 (7.39%, 17/230) was similar to that of the clinical *K. pneumoniae* isolates collected from the same district during 2010-2014 (7.01%, 23/328) (Lu et al., 2018). The protein showing the highest identity (88.90%) with FloR-T2 was a FloR protein (YP_005351917.1) identified in *Klebsiella pneumoniae*. Currently, FloRv was the FloR variant with the lowest identity (88.40%-91.80%) to other previously reported FloR proteins (He et al., 2015). FloR-T2 exhibited less identity with other FloR proteins than FloRv. Furthermore, FloR-T2 was shown to be one of the most divergent members of the FloR family, followed by FloRv (Figure S1).

It was interesting to find that the expression levels of *floR-T2* increased much more significantly in the host TL1285 or the recombinant than those of *floR-T1* when induced by the amphenicols. Using transcriptome sequencing, Lang et al. found that the expression of the *floR* gene of the *E. coli* plasmid pAR060302 increased 8-fold under the induction of florfenicol (Lang et al., 2012). Yinghui et al. also reported that the mRNA levels of the *floR* gene encoded by ICEAp12 on chromosomes increased in the presence of chloramphenicol (Li et al., 2018). However, the modulation mechanisms of mRNA expression of *floR* variants remain unclear. As reported by Yinghui et al. (Li et al., 2018), we also identified the translational attenuator region upstream of the *floR* variants. In addition, we found that the peptide encoding region of *floR-T1* was identical to those of pA060302 and ICEAp12 reported by Yinghui (Li et al., 2018) (Figure 3). It is known that the expression of chloramphenicol resistance genes, including *catA, cmlA* and *fexA*, could be induced by chloramphenicol, and this induction is mediated by translational attenuator structure at the post-transcriptional level (Stokes and Hall, 1991; Kehrenberg and Schwarz, 2004; Schwarz et al., 2004). However, considering that the stem-loop structure is distant to the RBS site of the *floR* gene, it is not clear whether this structure participates in the induced expression of the *floR* gene.

WGS result revealed that *floR-T1* and *floR-T2* of *P. aeruginosa* TL1285 were related with a novel *Tn4371*-like ICE. *Tn4371* is a 55-kb ICE that can be integrated into the *attB* site (5’T-TTTTCAT-3’) through a site-specific recombination process since the ends of the element can be detected covalently as a transfer intermediate (Merlin et al., 1999; Toussaint et al., 2003). The *Tn4371*-like ICEs are mosaic in structure and consist of Ti-RP4-like transfer systems, an integrase region, plasmid maintenance genes and accessory genes (Toussaint et al., 2003). Any ICE that encodes an integrase gene closely related to *intTn4371* (>70% protein homology) and has similar maintenance and transfer genes could be considered as a member of the *Tn4371* family (Ryan et al., 2009). The *Tn4371*-like ICEs carrying *floR* variants have been identified in the *P. aeruginosa* strains of different MLST types (such as ST308 and ST316) isolated from different samples of different countries. *P. aeruginosa* ST308 is a high-risk clone that can locally acquire resistance determinants from water-distribution system and was involved in a five-year outbreak in a French hospital between 2005 and 2010 (Jeanvoine et al., 2019). The variable region of these *Tn4371*-like ICEs also carried other ARGs like *blaNDM-1*, *tetA* and *msr(E)*. These findings indicate that the *Tn4371*-like ICEs might have emerged as a potential vehicle to mediate the spread of drug resistance genes in *P. aeruginosa* isolates.

**CONCLUSION**

In this study, we determined the prevalence of *floR* among 430 clinical isolates of *P. aeruginosa* and characterized two *floR* variants, *floR-T1* and *floR-T2*, in a *P. aeruginosa* strain TL1285. The *floR-T1* gene was the most prevalent variant in clinical *P. aeruginosa* strains. The *floR-T2* is a novel *floR* variant that showed less identities with the other *floR* proteins than FloRv. The mRNA levels of the two *floR* variants could be induced by florfenicol and chloramphenicol and the expression level of *floR-T2* was significantly higher than that of *floR-T1*. Inverted repeat sequences as well as stem-loop regions of the translational attenuators differed among the *floR* variants. The *floR-T1* and *floR-T2* of TL1285 were located on an incomplete novel *Tn4371* family ICE, while *floR-T2*-carrying ICEs were also identified in
other five P. aeruginosa genomes. These results indicate that Tn4371 family ICEs might be related with the dissemination of floR-T2 among P. aeruginosa strains.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

JL, HX, HYL, XL, KL, and HZ collected the strains. HML, WL, JC, XZhang, KS, and QL performed the experiments. QC, HLL, and XZhu analyzed the experimental results. CQ, WZ, and AL performed the bioinformatics analysis. CQ, TX, and QB co-led the writing of the manuscript. HZ, ZM, and QB designed the work. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflict of interest.

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