Identification of floR Variants Associated with Tn4371-Like Integrative and Conjugative Elements in Human Clinical Pseudomonas Aeruginosa Isolates

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Research

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Abstract

Background: Florfenicol is widely used to control respiratory diseases and intestinal infections in food animals. However, dramatic and serious florfenicol resistance in various clinical strains was reported. As a key resistance gene for florfenicol, *floR* has often been associated with mobile genetic elements. To analyze the potential transmission of *floR*, we investigated *floR* gene in *Pseudomonas aeruginosa* isolates from human clinical samples and characterize two *floR* variants, *floR-T1* and *floR-T2*.

Methods: Pooled genomic DNA sequencing and PCR were used to analyze the *floR* gene in *P. aeruginosa*. The *floR* variants were cloned, and the minimum inhibitory concentrations (MICs) were determined. Quantitative RT-PCR was used to compare the gene expression of the two *floR* variants in TL1285 with or without florfenicol/chloramphenicol. Whole-genome sequencing was used to identify the genetic context of the *floR* variants in TL1285.

Results: Three types of *floR* variants (designated *floR, floR-T1* and *floR-T2*) were identified in the clinical *P. aeruginosa* isolates, and *floR-T1* was the most prevalent variant. The positive rates of the *floR-T1* gene in the *P. aeruginosa* strains collected in 2008-2009 and 2015-2017 were 3.00% (6/200) and 7.39% (17/230), respectively. FloR-T2 exhibited less identity with other FloR proteins than FloRv. The two *floR* variants, *floR-T1* and *floR-T2*, in *P. aeruginosa* TL1285 were functionally active and located on a novel incomplete Tn*4371* family integrative and conjugative element (ICE). The expression of the two *floR* variants could be induced by florfenicol and chloramphenicol at different levels.

Conclusions: Two *floR* variants, *floR-T1* and *floR-T2*, were identified in a clinical *P. aeruginosa* strain. Tn*4371* family ICEs contribute to the dissemination of resistance genes among *P. aeruginosa*. Antimicrobial resistance could be transmitted from animal bacteria to human pathogens, posing a severe threat to public health.

Background

Florfenicol, a fluorinated thiamphenicol derivative, has been licensed in China since 1999 and has been widely used to control respiratory tract diseases and enteric infections in food-producing animals [1]. However, due to the inappropriate use of florfenicol to prevent or cure bacterial infections, florfenicol resistance has become increasingly serious in veterinary medicine [2]. 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* [3–5].

To date, seven florfenicol resistance genes (excluding variants), *floR*, *fexA*, *fexB*, *cfr*, *optrA, pexA* and *estDL136*, have been reported; [6–13] among them, *floR* is one of the main florfenicol resistance genes in gram-negative bacteria [14]. Several variants of the *floR* gene, including *pp-flo*, *cmlA*-like, *floRv* and *floSt*, have been documented, and most of them encode 404 amino acids. These *floR* variants are closely related to each other, and *floRv* from *Stenotrophomonas maltophilia* shares the lowest amino acid

identity (88.4%-91.8%) with the others excluding pp-flo [14]. The *floR* gene has been detected either on chromosomes or plasmids of various bacteria and has often been associated with mobile genetic elements and genomic islands.

As an opportunistic pathogen, *Pseudomonas aeruginosa* is a major cause of nosocomial infections, such as respiratory infections in debilitated patients and patients with cystic fibrosis, and is known for its resistance to antibiotics [15, 16]. Typically, *P. aeruginosa* is clinically resistant to chloramphenicol, but rifampicin-tobramycin conjugates have been shown to break the intrinsic resistance of *P. aeruginosa* to chloramphenicol in vitro or in vivo, thus expanding the therapeutic usefulness of this agent [17]. However, the *floR* gene carried by this pathogen may cause this strategy to fail when chloramphenicol is used. In this study, we screened the *floR* gene of clinical *P. aeruginosa* isolates collected in the years 2008–2009 and 2015–2018. As a result, a *P. aeruginosa* strain named TL1285 carrying two functionally active *floR* variants was identified.

Methods

Bacterial isolation and sequencing

A total of 430 clinical *P. aeruginosa* strains isolated from sputum, urine or blood samples of patients were collected at the First Affiliated Hospital of Wenzhou Medical University, Wenzhou, China. Among these isolates, approximately 200 strains were isolated from 2008–2009, and 230 strains were isolated from 2015–2017. 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 isolated periods, the genomic DNA was pooled as TL0809 and TL151617 and subsequently used for high-throughput sequencing by the Illumina paired-end strategy. Genomic DNA of TL1285 was also sequenced by Pacific Bioscience sequencers at Annoroad Gene Technology Co., Ltd. (Beijing, China).

Genome assembly, annotation, and bioinformatics analysis

Genome assembly of pooled DNA sequencing was performed using megahit [18], and contigs less than 400 bp were discarded. The complete genome of *P. aeruginosa* TL1285 was assembled using Canu [19] with long reads obtained from PacBio. Error correction of tentative complete circular sequence was performed using Pilon [20] with short read sets derived from Illumina sequencing. Open reading frames (ORFs) of pooled DNA sequences were predicted using Prodigal [21] with default parameters. Using the antibiotic resistance genes of the CARD [22] and ResFinder [23] 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 [24] and then verified by BLASTP searches against the UniProtKB/Swiss-Prot [25] and RefSeq [26] databases. Annotation of mobile genetic elements was carried out using online databases including ISfinder [27], INTEGRALL [28], and the Tn Number Registry [29].

PCR amplification and cloning of the floR gene

Genomic DNA of each of the 430 isolates was screened for the *floR* gene using a PCR method with the primers listed in Table S1. PCR amplification was carried out under the following conditions: initial denaturation of 5 min at 95 °C; 35 cycles of denaturation (94 °C for 45 s), annealing (58 °C for 45 s), and extension (72 °C for 90 s); and a final extension step at 72 °C for 10 min. The *floR-T1* and *floR-T2* gene sequences with promoter regions were amplified from *P. aeruginosa* TL1285 and ligated into pUCP24, and the recombinant plasmids were transformed into *P. aeruginosa* PAO1.

Antimicrobial susceptibility testing

Minimum inhibitory concentrations (MICs) of 17 antimicrobial agents were determined using the agar dilution method and interpreted in accordance with the guidelines of the Clinical and Laboratory Standards Institute (CLSI document M100-S27, 2017).

Comparison of the expression of *floR-T1* and *floR-T2* in *P. aeruginosa* TL1285

Quantitative reverse transcription PCR (qRT-PCR) was used to investigate the expression of the *floR* variants in TL1285 in the presence/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 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 qPCR primers (Table S1).

Results And Discussion

Identification of the *floR* variants

To investigate the prevalence of the *floR* gene among clinical *P. aeruginosa* isolates, bacterial genomic DNA was pooled according to the isolated periods as TL0809 (200 *P. aeruginosa* strains isolated from 2008–2009) and TL151617 (230 *P. aeruginosa* strains isolated from 2015–2017). The Illumina sequencing of TL0809 and TL151617 generated 1.4×10^8 and 4.6×10^8 filtered reads with average depths of ~ 20 and ~ 50, respectively. The reads of TL0809 and TL151617 were assembled to generate 20,205 and 41,932 contigs, with N50 values of 3,550 and 2,316 bp in length, respectively. Using the *floR* gene

(AF231986) as a reference, three types of *floR* variants (the reference *floR, floR-T1* and *floR-T2*, named in this study) were identified in the two pooled sequencing libraries, of which TL0809 contained all three and TL151617 contained only two *floR* variants (the reference *floR* and *floR-T1*) (Table 1).

Table 1.

The Abundance and PCR positive rate of the floR variants

	floR variants	Identity	Match length (aa)	Abundance[b]	PCR positive rate
TL050607	*floR*[a]	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	17/230 (7.39%)

[a] reference floR (AF231986)

[b]Abundance = average fold of gene * 4 * 100 / average fold of 16S rRNA

Using homology tree analysis, the amino acid identities of FloR-T1 and FloR-T2 with the known *floR* proteins ranged from 90.80–100% and 86.10–88.90%, respectively (Fig. 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*. FloRv was the *floR* variant with the lowest identity (88.40% − 91.80%) to other FloR proteins reported so far. However, 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 (Fig. S1). Therefore, *floR-T2* is a novel *floR* variant identified in this study.

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 1). The positive rates were consistent with the abundance 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 increased compared with that from 2008–2009 (3.00%, 6/200), which was also similar to the positive rate of the *floR* gene (7.01%, 23/328) of the human clinical *K. pneumoniae* isolates collected from the same district [5]. Zhan et al. also reported a dramatic increase in florfenicol-resistant invasive nontyphoidal *Salmonella* (iNTS) in China between 2007 and 2016 [3]. The results indicate that antimicrobial resistance could be transmitted from animal bacteria to human pathogens, which might pose a severe threat to public health.

Antimicrobial susceptibility of *P. aeruginosa* TL1285 and the *floR* clone

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 both chloramphenicol and florfenicol (Table 2). Cloned *floR-T1* (pUCP24-*floR-T1*/PAO1) increased the MICs for chloramphenicol and florfenicol by 2- and 4-fold, and the MICs of chloramphenicol and florfenicol against the transformant

carrying *floR-T2* (pUCP24-*floR-T2*/PAO1) increased 4- and 8-fold, respectively. The results indicate that *floR-T1* and *floR-T2* of *P. aeruginosa* TL1285 are functionally active.

Table 2
Antimicrobial susceptibility of *P. aeruginosa* TL1285

Antibiotics	MIC (μg/mL)
Ampicillin	1024
Ceftazidime	< 1
Levofloxacin	< 0.5
Cefpyridine	4
Minocycline	64
Chloramphenicol	128
Florfenicol	256
Ciprofloxacin	2
Azithromycin	32
Fosfomycin	256
Tigecycline	4
Colistin	< 1
Erythromycin	256
Nalidixic acid	> 1024
Gentamicin	> 1024
Kanamycin	64
Streptomycin	> 1024

Expression of the *floR* variants

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 [30]. Yinghui *et al* also reported that the mRNA levels of the *floR* gene encoded by ICEApl2 on chromosomes increased in the presence of chloramphenicol [31]. In this work, we performed qRT-PCR to explore the expression of the two *floR* variants with or without florfenicol and chloramphenicol induction. The mRNA levels of *floR-T2* in *P. aeruginosa* TL1285 increased 78-fold and 56-fold in the presence of florfenicol and

chloramphenicol, while the mRNA levels of *floR-T1* only increased 1.2-fold and 1.6-fold in the presence of florfenicol and chloramphenicol, respectively (Fig. 1).

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 a translational attenuator at the post-transcriptional level [8, 13, 32]. The translational attenuators that located immediately upstream of these chloramphenicol resistance genes consist of a single pair of inverted repeated sequences, IR1 and IR2, as well as a reading frame of a 6–9 aa short peptide [13]. IR1 and IR2 were able to form a stable stem-loop structure blocking the resistance gene-associated ribosome binding site (RBS). Yinghui *et al* reported that the region upstream of the *floR* genes also contains a well-conserved similar attenuator sequence [31]. However, the stable stem-loop structure of the attenuator sequence did not overlap with the RBS site of the *floR* gene. Interestingly, we found that the peptide sequence of the *floR*-associated attenuator sequence in pA060302 and ICEApl2 reported by Yinghui [31] was identical to that of *floR-T1* but different from that of *floRv* or *floR-T2* by 3 amino acids (Fig. 2). Furthermore, the attenuator sequences of *floR-T2* and *floRv* encode an identical peptide sequence, although their nucleotide sequence varies by one base. The inverted repeats of the attenuators of these *floR* genes were also different, and the stem-loop structures formed in distinct stable states. The genes *floR-T2* and *floRv* have the most stable structure among the four variants. 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.

floR-T2 encoded in a Tn*4371*-like ICE

Whole genome sequencing (WGS) of *P. aeruginosa* TL1285 only produced a circular 6,609,407 bp chromosome with an average GC content of 66.06% encoding 5,611 ORFs. Multiple antimicrobial resistance genes, 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 antimicrobial resistance phenotype was in accordance with the genotype (Table 2). The florfenicol-resistant genes *floR-T1* and *floR-T2* were embedded in an 86-kb Tn*4371*-like integrative and conjugative element (ICE) (Fig. 3). Tn*4371* is a 55-kb ICE that can be integrated into the *attB* site (5'-TTTTCAT-3') through a site-specific recombination process since the ends of the element can be detected covalently as a transfer intermediate [33, 34].

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 to carry *floR-T2*. MLST analysis of the five *P. aeruginosa* chromosomes together with *P. aeruginosa* TL1285 showed that WPB099, WPB100, WPB101, PASGNDM345 and PASGNDM699 belonged to ST308, while TL1285 belonged to ST316. *P. aeruginosa* ST308 is a high-risk clone that can locally acquire resistance determinants originated from the water-distribution system and caused a five-year outbreak in

patients in a French hospital [35]. Interestingly, these *floR-T2*-carrying strains came from different resources. *P. aeruginosa* WPB099, WPB100 and WPB101 were isolated from hospital wastewaters in Singapore. *P. aeruginosa* PASGNDM345 and PASGNDM699 were derived from patient sputum in Singapore, while *E. cloacae* AR_038 and TL1285 were isolated from patient sputum in the United States and China, respectively. Whole genome alignment of six *P. aeruginosa* strains suggested that the strains shared high identity, and their differences were mainly focused on some genomic islands (Fig. 3). The Tn*4371*-like ICE carrying *floR-T2* in TL1285 was also partially present in these five *P. aeruginosa* strains. It may 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, so the precise genetic environments around *floR-T2* could not be described.

Tn*4371*-like ICEs are mosaic in structure and consist of Ti-RP4-like transfer systems, an integrase region, plasmid maintenance genes and accessory genes [34]. Comparative analysis of the Tn*4371*-like ICE regions of six *P. aeruginosa* strains revealed that the plasmid maintenance system (*repA, parA* and *parB*) and conjugational transfer systems were conserved among these sequences (Fig. 4). The variable region between the *traF* and *traR* genes, which encoded a biphenyl catabolic *bph* gene cluster in Tn*4371* (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 was inserted between *czcD* and *lysR*, which encode bla_{NDM-1}, *msr(E)* and *floR-T2* genes. The insertion fragment was flanked by 695 bp direct repeats, which were also present in WPB099, WPB100 and WPB101, whereas it showed no similarity to the existing IS element [36]. 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 results indicate that Tn*4371*-like ICEs might have emerged as a potential vehicle to mediate the spread of drug resistance genes in *P. aeruginosa* isolates.

Any ICE that encodes an integrase gene closely related to int_{Tn4371} (> 70% protein homology) and has similar maintenance and transfer genes could be considered a member of the Tn*4371* family [37]. The integrase genes (*int*) of PASGNDM345, PASGNDM699, WPB99, WPB100 and WPB101 were identical and shared 78% identity with that of Tn*4371*. However, no homologue of int_{Tn4371} was found in TL1285 (Fig. 4). Tn*4371* family ICEs could be integrated into the genome through an 8-bp *attB* site, generating direct repeat *attL* and *attR* element chromosomal junctions [33]. In PASGNDM345, PASGNDM699, WPB99, WPB100 and WPB101, 8-bp repeats (5'-TTTTTTGT-3') were identified on both extremities of the ICE region. However, in TL1285, only *attR* was found (Fig. 4). The *noc* gene upstream of *repA* in TL1285 was truncated by a novel Tn*402* family transposon. The transposon is formed by IS*Cfr1* and In*2* carrying a single *aadA5* cassette embedded downstream of the *tnpR* gene of Tn*1013*, and this Tn*402* family transposon was surrounded by 37-bp imperfect inverted repeats (Fig. S2).

Inverse PCR using primers P1, P2 and P3 (Fig. 4) was performed to detect whether the ICE in TL1285 could generate a circular extrachromosomal form, but no positive results were observed. Taken together, we speculate that the ICE in TL1285 is an incomplete member of the Tn*4371* family and may have lost the ability to excise and integrate. The insertion of the Tn*402* family transposon leads to the loss of the upstream sequence of the *nor* gene, including the integrase gene *int* of ICE.

Conclusion

In conclusion, three *floR* variants, *floR*, *floR-T1* and *floR-T2*, were identified in clinical *P. aeruginosa*, and *floR-T1* was the most prevalent variant. The gene *floR-T2* was a novel *floR* variant identified in this study that showed less identity with other FloR proteins than FloRv. One *P. aeruginosa* strain, TL1285, carrying *floR-T1* and *floR-T2* was identified, and the whole genome of this strain was sequenced. The *floR-T2* gene of TL1285 was located on an incomplete novel Tn*4371* family ICE, and similar ICEs were also identified in five other *floR-T2*-carrying *P. aeruginosa* genomes. The expression of the two *floR* variants could be induced by florfenicol and chloramphenicol at different levels. Our results indicate that Tn*4371* family ICEs may cause the dissemination of *floR-T2* and other drug resistance genes in *P. aeruginosa*. Antimicrobial resistance could be transmitted from animal bacteria to human pathogens, posing a severe threat to public health.

Abbreviations

ICE, Integrative and conjugative element; BLAST, The Basic Local Alignment Search Tool; MIC, Minimum Inhibitory Concentration; qRT-PCR, Quantitative reverse transcription PCR; ORFs, Open reading frames; RBS, ribosome binding site; PCR: polymerase chain reaction. WGS, Whole genome sequencing.

Declarations

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Authors' contributions

JL, HX, HYL, XL, KL and HZ collected the strains. HML, WL, JC, XYZ, KS and QL performed the experiments. QC, HLL and XZ analyzed the experimental results. CQ, WZ and AL performed the bioinformatics analysis. CQ, TX and QB co-led the writing of the manuscript. CQ and QB designed the work. All authors read and approved the final manuscript

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Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files. The data related to the paper are deposited in the NCBI GenBank. The accession numbers for *P. aeruginosa* TL1285 chromosome is CP053390.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors have no competing interests to declare.

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Figures

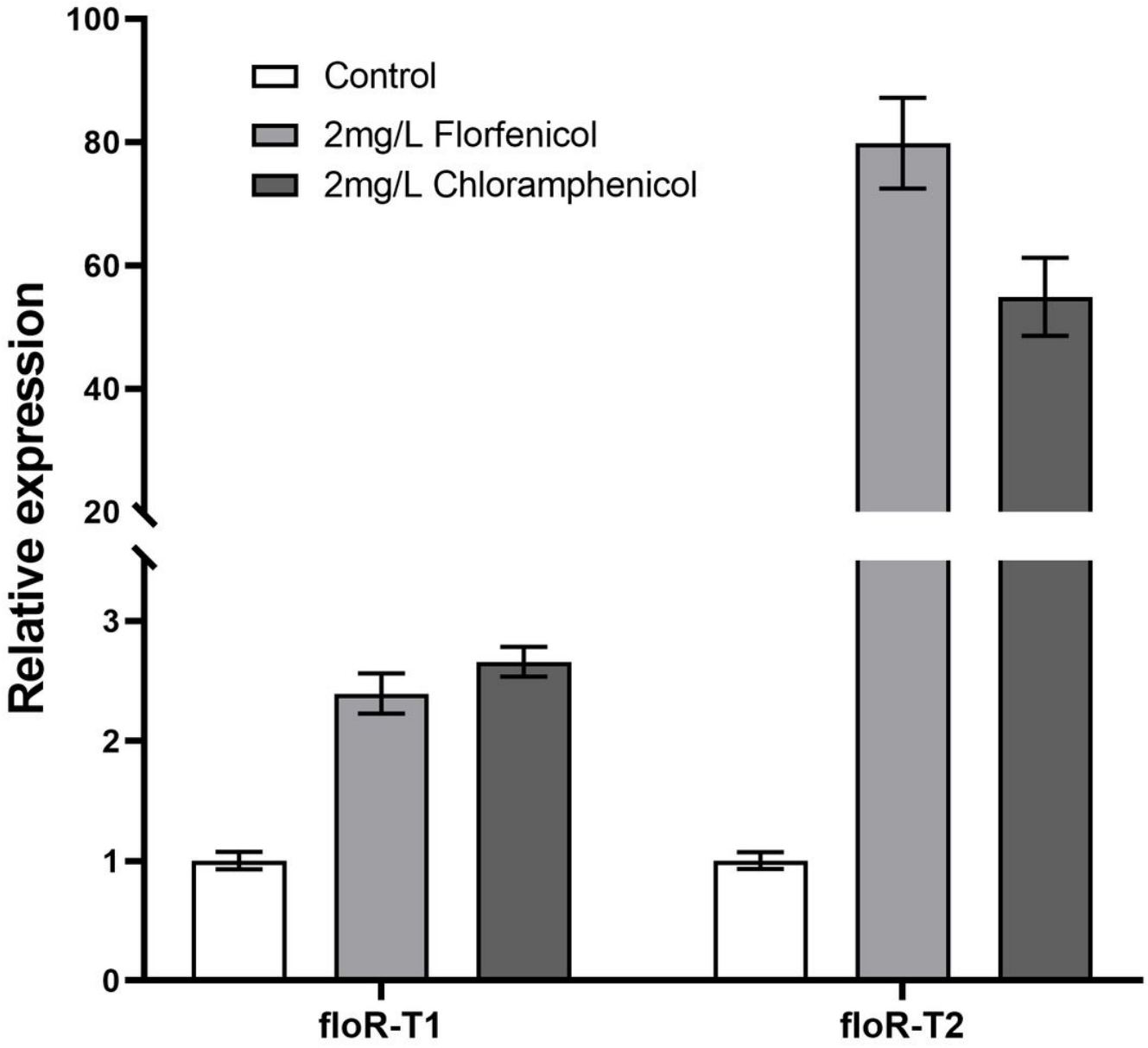

Figure 1

Expression of the floR variants in TL1285 with or without florfenicol or chloramphenicol induction.

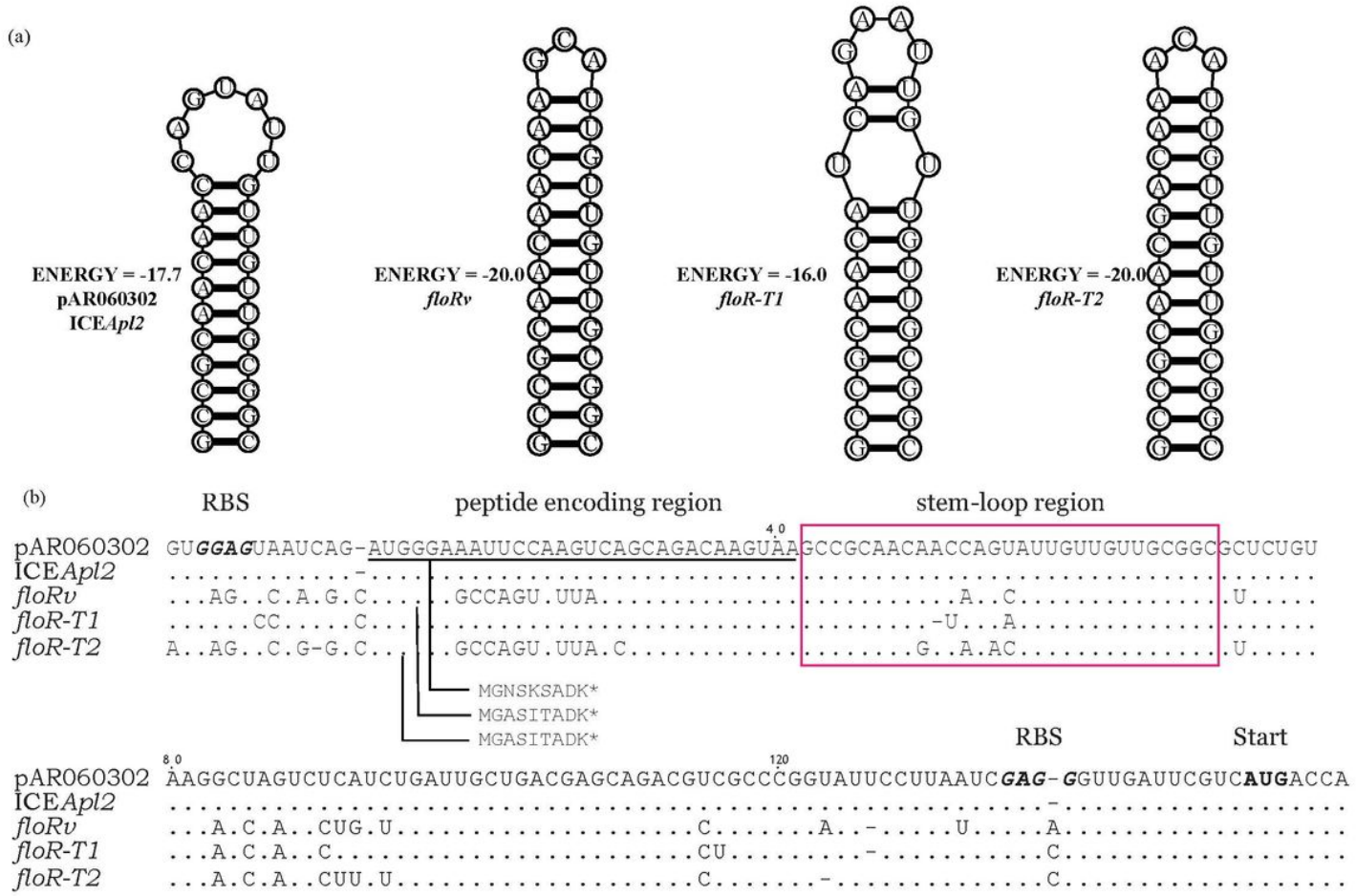

Figure 2

Comparison of sequences upstream of the floR variants. (a) The stable mRNA secondary structure of floR variants formed by inverted repeat sequences boxed in (b). (b) The attenuator of floR variants consists of a peptide encoding region (bold type) and stem-loop region (boxed). The start codons and ribosome binding sites (RBS) of short peptide and floR are labeled and displayed in bold type letters.

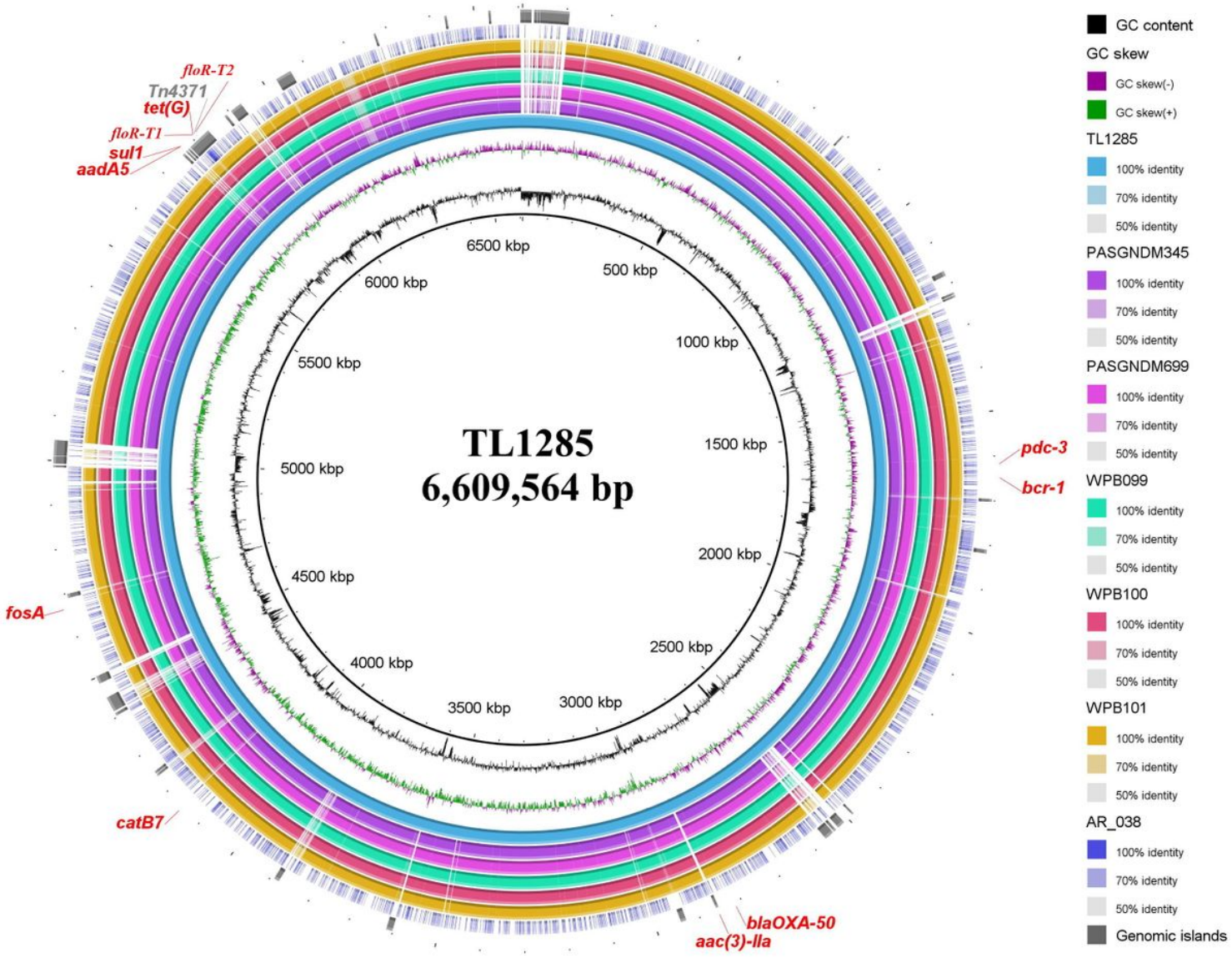

Figure 3

Sequence conservation among P. aeruginosa TL1285 and 6 other genomes carrying floR-T2. From the 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; Circle 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 of TL1285.

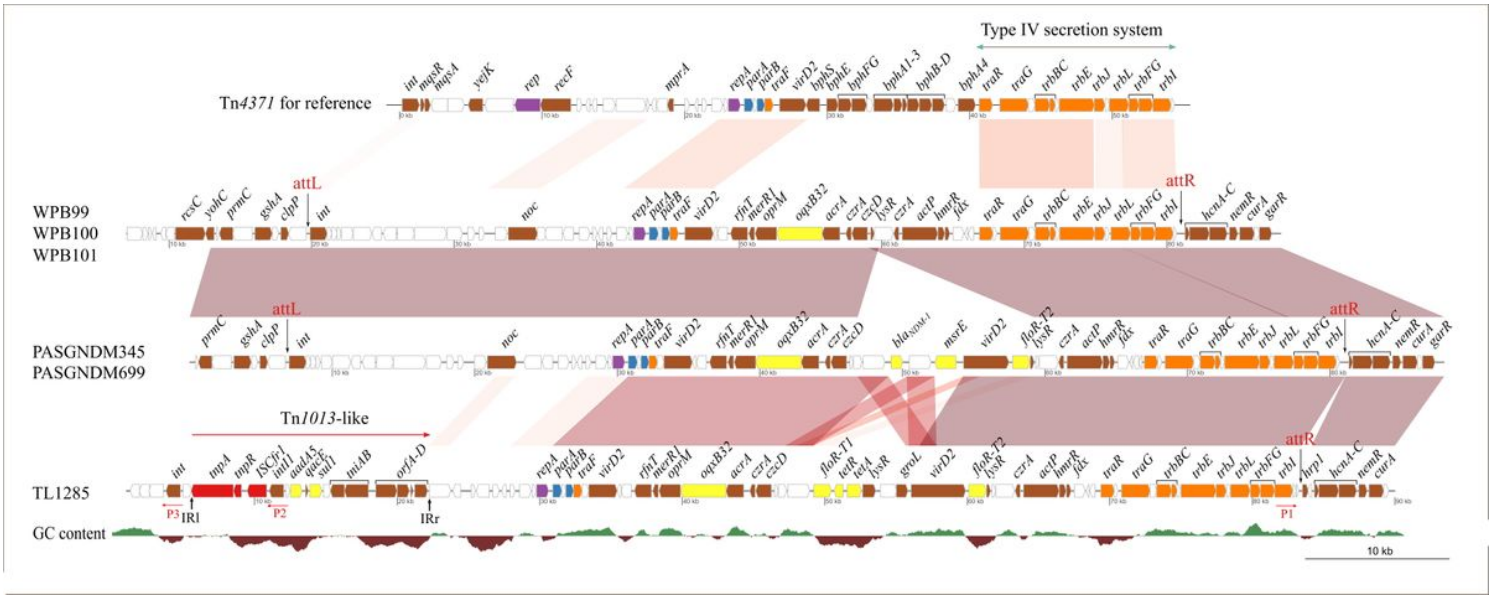

Figure 4

Comparative genomic analysis of ICE region of TL1285 and 5 other floR-T2-carrying P. aeruginosa genomes. 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: hypothesis proteins.

Supplementary Files

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- FigureS2.jpg
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- TableS1.docx