

Florfenicol-resistant, Rabbit-derived *Providencia Rettgeri* Isolate With 4 Copies of *floR* Genes in a Tandem-repeat Plasmid

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Research

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Abstract

Background: This work aimed to investigate the resistance mechanism to antimicrobial agents (especially florfenicol) exhibited by *Providencia* isolates obtained from various sources.

Methods: The polymerase chain reaction (PCR) was performed to detect the distribution of the florfenicol resistance genes. Conjugation, S1-PFGE (S1 nuclease pulsed-field gel electrophoresis), sequencing and other molecular methods were employed to analyze the transferability and structure of the resistance gene-encoding plasmids.

Results: It showed that the human pathogens exhibited a wider resistance spectrum, while the animal bacteria had higher MIC values for most antibiotics analyzed. Of the seven florfenicol resistance genes (*floR*, *fexA*, *fexB*, *optrA*, *estDL136*, *cfp* and *pexA*) screened, only *floR* was identified in both animal (5/10, 50%) and human bacteria (3/13, 23.1%), and none of them was detected in freshwater fish isolates (0/22). The *floR*-positive strains exhibited high MIC levels for florfenicol (≥ 64 mg/L). The whole genome sequencing of the multidrug-resistant *Providencia rettgeri* strain R39 demonstrated that it carried four plasmids, with two larger plasmids encoding 5 copies of the *floR* genes. The largest plasmid, **pR39-33** (32,936 bp) with 4 *floR* genes, was composed of 4 copies of the second large plasmid **pR39-8** which encoded 8 ORFs (open reading frames) including a *floR* and two mobilization genes (*mobAC*) in the form of tandem repeat. It indicated that the *floR* gene represented the primary mechanism underlying the florfenicol resistance of the *Providencia* isolates.

Conclusions: This report is the first to describe an antibiotic-resistant plasmid composed of four copies of another plasmid sequence in the form of a tandem repeat.

Background

The *Providencia* species belong to Enterobacteriaceae; unlike many other bacteria of this family, these species are considered to be uncommon causes of infections. The genus *Providencia* consists of eight species that have been isolated from a wide variety of environments, such as wastewater, oceans, food animals and human clinical isolates. *Providencia* species have been isolated from numerous animal sources worldwide, including penguin feces in German zoos, sea turtles in the Mediterranean [1], shark mouths in Brazil [2], and so on. Among the species in the genus *Providencia*, *P. stuartii*, *P. rettgeri* and *P. alcalifaciens* are the most common human opportunistic pathogens. *P. stuartii* strains have been reported to cause bacterial pericarditis [3], meningitis [4] and peritonitis [5]. *P. rettgeri* has been isolated from patients with urinary tract infections [6], nephrocutaneous fistula [7], and the bacteria has been obtained from the soft tissue of the toe of a patient suffering from diabetes [8]. *P. alcalifaciens* is the most frequently reported causative agent of diarrhea outbreaks in both humans [9] and animals [10–12]. With the overuse of antibiotics in human clinical and agricultural practice, an increasing number of multidrug-resistant *Providencia* strains have been isolated [13–16]. *Providencia stuartii* was observed to simultaneously carry the *bla*_{VIM-2}, *bla*_{PER-1} and *armA* genes [14, 17]. Carbapenem-resistant *Providencia stuartii* with CTX-M-2 and AmpC [15] and extended-spectrum β -lactamase (ESBL)-producing *Providencia* spp [16] have also been reported.

Florfenicol is a derivative of chloramphenicol that is utilized to treat bacterial infections in animals. However, compared with chloramphenicol, florfenicol is a special veterinary drug that chloramphenicol acetyltransferase cannot deactivate [18, 19]. Florfenicol is able to bind the 50S ribosomal subunit to inhibit the protein biosynthesis of the bacteria. Because of the wide antimicrobial spectrum, good absorption, and wide distribution in the animal body with few side effects, florfenicol has been widely utilized in veterinary medicine and animal farming [18, 20]. Accompanying the use of florfenicol, an increasing number of florfenicol-resistant bacteria, such as *Pasteurella multocida* [21], *Actinobacillus* [22] and *Mannheimia haemolytica* [23], have emerged. To date, a number of florfenicol resistance-associated genes, including *fexA* [24], *fexB* [25], *floR* [26], *cfr* [27], *optrA* [28, 29], *pexA* [30], *estDL 136* [31] and their variants, have been identified. However, the resistance characteristics of these bacteria, especially the molecular resistance mechanism of *Providencia rettgeri* to florfenicol, have not been elucidated. In this work, the florfenicol resistance of 45 *Providencia* isolates of different sources was analyzed, and the genome structure of *Providencia rettgeri* with 5 copies of *floR* genes was characterized.

Materials And Methods

Bacterial strains and antimicrobial susceptibility testing

The bacterial isolates examined in this work are listed in Table 1 and Table S1. Among the 45 nonduplicate isolates of *Providencia*, 13 were isolated from human clinical samples at the Affiliated Hospital of Wenzhou Medical University in Wenling, China, from 2015 to 2017. Ten and 22 isolates were isolated from fecal samples of domestic animals and freshwater fish intestinal contents, respectively, in Wenzhou, China, in 2016 and 2018. The isolates were first identified by the Vitek-60 microorganism autoanalysis system (BioMerieux Corporate, Craaponne, France) and subsequently by 16S rRNA gene sequencing comparison of the *Providencia* isolates with those in the NCBI nucleotide database.

The minimum inhibitory concentrations (MICs) were determined by the agar dilution method, and the results were interpreted according to breakpoints suggested by the Clinical and Laboratory Standards Institute (CLSI, 2019). Meanwhile, the resistance threshold values (32 mg/L) for florfenicol were chosen according to the published values obtained for *Escherichia coli* [32]. *E. coli* ATCC 25922 and *E. coli* DH5a were employed as reference strains in each antimicrobial susceptibility test.

Polymerase chain reaction analysis

The template genomic DNA of the isolates was extracted by AxyPrep Bacterial Genomic DNA Miniprep kits (Axygen Scientific, Union City, CA, USA). The primers for the 7 florfenicol resistance-associated genes (*fexA*, *fexB*, *floR*, *cfr*, *optrA*, *pexA* and *estDL 136*) are presented in Table 2. The PCR products were purified using a MinElute PCR Purification kit (QIAGEN China, Shanghai, China) and sequenced by Sanger sequencing (Annoroad Gene Technology Co., Ltd., Beijing, China). The nucleotide sequences of the PCR products were analyzed and compared with those in the NCBI nucleotide database using the BLAST program (<http://www.ncbi.nlm.nih.gov/BLAST>).

Genome sequencing, assembly and bioinformatic analyses

The whole genome sequence of *P. rettgeri* R39 DNA was sequenced by Illumina HiSeq 2500 and Pacific Bioscience sequencers at Annoroad Gene Technology Co., Ltd. (Beijing, China). The sequence contigs were obtained using SOAPdenovo software to assemble the reads. When a gap was encountered, we remapped the short reads or sequenced the PCR product of the gap. Potential open reading frames (ORFs) were predicted using Glimmer3.02 with default parameters (<http://ccb.jhu.edu/software/glimmer>) and annotated by BLASTX (<https://blast.ncbi.nlm.nih.gov>) with an e-value threshold of 1e-5. Gview (<https://server.gview.ca/>) was utilized to construct basic genomic features that were subsequently employed in comparative genomic analysis.

Collection and processing of plasmid sequences

We compared the sequence of pR39-8 against the NCBI nucleotide database with a cutoff value (max score) of approximately 8700. The sequences with $\geq 80\%$ identity with pR39-8 were retained as candidates for later orthologous analysis using BLASTP [33] and InParanoid [34]. The other bioinformatic tools used in this work were written with Python and Biopython scripts.

S1-PFGE and restriction enzyme digestion of plasmids

Pulsed-field gel electrophoresis (PFGE) was performed as described previously [35]. *P. stuartii* genomic DNA in agarose blocks was digested with S1 nuclease (Takara, Dalian, China). A contour-clumped homogeneous electric field (CHEF) method was used for S1-PFGE on a CHEF DRII system (Bio-Rad Laboratories, Munich, Germany). The resulting fragments were resolved in a 1.0% agarose gel run at 6 V/cm in 0.5*TBE (Tris-borate-EDTA) electrophoresis buffer at 14°C, with switch times ramping from 3 s to 30 s for 14 hours.

Plasmid DNA from *P. stuartii* R39 was isolated using the QIAGEN Plasmid Midi Kit (QIAGEN GmbH, Germany). Plasmid bands from agarose gels were isolated using the Omega E.Z.N.A. Ultra-Sep Gel Extraction Kit (Omega Bio-tek, Norcross, USA) according to the protocol. The fingerprinting of the isolated plasmid DNA was confirmed by digestion with the restriction enzymes *Bam*HI (Takara, Dalian, China) and/or *Eco*RV (Takara, Dalian, China) at 37°C for 2 h, and agarose gel electrophoresis was performed to determine the sizes of the resulting restriction fragments.

Cloning of the open reading frame of the *floR* gene with its promoter region

To confirm the function of the *floR* genes, we PCR-amplified and cloned the ORF of the *floR* gene with its predicted promoter region (<http://www.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb>) from R39 into the pUC18 vector. The primers (Table 2) were designed using Primer Premier 5.0 and synthesized by Shanghai Sunny Biotechnology Co., Ltd. (Shanghai, China). The plasmid containing *floR* (pUC18-*floR*) was transformed into *E. coli* DH5 α using the calcium chloride method, and bacterial colonies were grown on Luria-Bertani agar plates supplemented with ampicillin (100 mg/L). Recombinants (pUC18-*floR*/DH5 α) were confirmed by sequencing and MICs against the corresponding antibiotics.

Plasmid curing

P. rettgeri R39 was cultured on LB plates at 37°C overnight. Colonies were selected and cultured in 3 mL LB broth in a vibrating incubator (37°C, 200 r/min) for 16 h, and 60 μ L of the cultures were then transferred to 3

mL LB broth supplemented with a sublethal concentration (16 mg/L) of sodium dodecyl sulfate (SDS) (Sigma, USA) and incubated in a vibrating incubator (43°C, 200 r/min) for 20 h. Later, 60 µL of the cultures were transferred to 3 mL of LB broth and cultured in a vibrating incubator (37°C, 200 r/min) for 16 h. The last two procedures were performed once and a second time. In every 2-3 rounds of the aforementioned procedures, the culture was checked to determine whether the plasmid was cured by the following procedures. The cultures were diluted in 0.9% normal saline, plated onto LB plates and incubated at 37°C for 24 h. Each colony was streaked on two LB plates, of which one was supplemented with 32 mg/L florfenicol, while the other was free of antibiotics. The colonies that grew on plates free of florfenicol and failed to grow on plates with florfenicol were defined as putative plasmid-cured derivatives. The physical loss of the plasmid in the cured derivative was further confirmed by PCR of the *floR* gene, antimicrobial testing, plasmid extraction and agarose gel electrophoresis.

Nucleotide sequence accession number

The nucleotide sequences for the plasmids pR39-8 and pR39-33 and the whole chromosome sequence of *P. rettgeri* R39 were submitted to DDBJ/EMBL/GenBank under accession numbers CP066317, CP066316 and CP066315, respectively.

Results

Antibiotic susceptibility testing and resistance gene screening of *Providencia* strains

A total of 45 nonredundant strains of five *Providencia* species were analyzed, and the strains included 29 *Providencia rustigianii* isolates, 5 *Providencia alcalifaciens* isolates, 5 *Providencia stuartii* isolates, 4 *Providencia rettgeri* isolates, and 2 *Providencia vermicola* isolates. The bacteria from 22 freshwater fish were all *P. rustigianii* isolates, while the 2 *P. vermicola* isolates were obtained from the animal specimens only. Thirteen isolates from patients were species of *P. alcalifaciens*, *P. rettgeri* and *P. stuartii* (Table S1).

The MIC results for 15 antibiotics indicated that the 45 isolates had higher resistance rates to levofloxacin (77.8%, 35/45) and tetracycline (53.3%, 24/45) and lower resistance rates to chloramphenicol (35.6%, 16/45), imipenem 14 (31.1%, 14/45), norfloxacin (24.4%, 11/45) and florfenicol (17.8%, 8/45), while more than 90% of the isolates were sensitive to the other 9 antibiotics, especially meropenem with all the strains sensitive to it. The clinical isolates had a wider resistance spectrum and showed resistance to 13 antibiotics (ranging from 7.7% to 100%). The animal isolates showed resistance to 8 antibiotics (ranging from 10.0% to 80.0%), while the freshwater fish bacteria only showed resistance to 5 antibiotics (ranging from 4.5% to 50.0%). Notably, although the clinical strains showed a wider resistance spectrum, the animal strains showed higher resistance rates than the clinical strains in 5 of 9 antibiotics, which they both showed resistance to, especially to florfenicol (the animal strains with a resistance rate of 50%, while the human clinical isolates was only 23.1%) (Table 3, Table S2).

Detection of the florfenicol resistance genes demonstrated that of the 7 genes (*floR*, *fexA*, *fexB*, *optrA*, *estDL136*, *cfr* and *pexA*) screened, only the *floR* gene yielded positive results in both human (3/13, 23.1%) and animal bacteria (5/10, 50%). The *floR*-positive strains had high MIC levels for florfenicol (> 32 mg/L) and

chloramphenicol, while the *floR*-negative isolates showed notably lower MIC levels (with all of them exhibiting MIC levels < 8 mg/L) (Table S2).

General features of the *P. rettgeri* R39 genome

To analyze the molecular mechanism governing the florfenicol resistance of the bacteria from animal sources, the whole genome of *P. rettgeri* isolate R39, which exhibited the highest florfenicol MIC level (Table S2), was sequenced. The R39 genome was determined to consist of a circular chromosome and 4 circular plasmids. The chromosome was 4,502,622 bp in size with 40.15% GC content encoding 4,029 ORFs. The four plasmids were named pR39-33 (32,936 bp, 32 ORFs), pR39-8 (8,234 bp, 8 ORFs), pR39-2.8 (2,888 bp, 6 ORFs) and pR39-2.7 (2,705 bp, 3 ORFs), respectively (Table 4, Fig. 1). The whole genome was observed to encode 9 resistance genes, of which 4 (*aadA1*, *sat2*, *catB2* and *drfA1*) were located on the chromosome, while the other 5 *floR* genes were located on the plasmids, with 4 *floR* genes on pR39-33 and one *floR* gene on pR39-8 (Fig. 1a, Fig. 1b). According to the drug susceptibility results, the drug resistance gene profile was consistent with the drug resistance spectrum of the bacterium (Table S2).

pR39-33, a plasmid composed of four copies of the pR39-8 sequence

PFGE analysis showed that R39 contained one plasmid of approximately 33 kb in length (Fig. 2a), while in the initial sequencing assembly, we only obtained the circular chromosome and three circular plasmids pR39-8, pR39-2.8, pR39-2.7 and a contig measuring approximately 25 kb in length. Annotation of the 25-kb contig sequence demonstrated that it contained three copies and more sequences of pR39-8 in the form of a tandem repeat. To determine if the largest plasmid was composed of four copies of pR39-8 sequences, the restriction physical map of the largest plasmid was analyzed. The physical map of pR39-8 demonstrated that it contained *EcoRV* (at position 4,381) and *BamHI* (at position 7,937) restriction sites 3,556 bp apart. When the plasmid was digested with both *BamHI* and *EcoRV*, two fragments of 3,556 bp and 4,678 bp were produced. We digested the plasmids extracted from R39 with *BamHI* or *EcoRV* alone, or with the combination of *BamHI* and *EcoRV*, and the results were obtained as predicted. When digested with *BamHI* or *EcoRV* alone, a single band of approximately 8 kb was obtained. When digested with *BamHI* + *EcoRV*, two bands of approximately 3.5 kb and 4.5 kb were observed (Fig. 2b). The digestion results of the recovered pR39-33 with *BamHI* or *EcoRV* alone or with both *BamHI* and *EcoRV* also yielded the expected results (Fig. 2c). This result confirmed that the structure of pR39-33 consisted of a tandem repeat of 4 copies of pR39-8 sequences.

The *floR* genes encoded on the plasmids

The plasmids (pR39-33 and pR39-8) cured R39 derivative (R39ΔpR39-33ΔpR39-8) exhibited notably decreased florfenicol MIC values. The florfenicol MIC decreased more than 128-fold from > 512 mg/L (the wild-type strain R39) to 4 mg/L (the plasmids cured R39, R39ΔpR39-33ΔpR39-8) (Table 5). The recombinant *floR* ORF together with its promoter region (pUC18–pro-*floR*/DH5α) conferred a much higher MIC level to florfenicol (256 mg/L) than the recipient *E. coli* DH5α (8 mg/L) (Table 5).

Comparative genomic analysis of the *floR* gene encoding plasmids

As mentioned above, R39 carried 2 resistant plasmids (pR39-33 and pR39-8), and pR39-33 is composed of a tandem repeat of four copies of the pR39-8 sequence. pR39-8 encoded 7 complete ORFs and a truncated insertion sequence (Δ IS91). These seven genes included a *floR* gene, 2 replication-related genes (*repA* and *repB*), 2 mobilization-related genes (*mobA* and *mobC*) and 2 hypothetical protein genes. The *floR* gene was related to a transposon-like structure (Δ IS91-hp-*floR*).

The five plasmids with the highest similarity (> 90% identity and > 83% coverage) to pR39-8 were retrieved from the NCBI nucleotide database (<https://www.ncbi.nlm.nih.gov/nucleotide/>). These plasmids have five or more genes that are the same as those encoded on pR39-8. The six plasmids described in this study were from bacteria of four different genera. Specifically, both plasmid pRCADGH-1 and plasmid pCCK381 were from *Pasteurella multocida*, the plasmids pM3446F and unnamed1 were from *Actinobacillus pleuropneumoniae*, pMh1405 is a *Mannheimia haemolytica* plasmid, and pR39-8 described in this work was from a strain of *P. rettgeri*. Of the five plasmid sequences obtained from the database, pRCADGH-1 exhibited the same sequence as pR39-8, but the plasmids were from two different genera, that is, *Pasteurella multocida* and *Providencia rettgeri*, respectively (Fig. 3, Table S3).

Discussion

In this study, *Providencia* strains isolated from different sources showed different resistance spectra of antibiotics tested. Although human bacteria are resistant to more antibiotics than animal or freshwater fish bacteria, animal *Providencia* strains showed higher resistance rates against some of the antibiotics, especially quinole (norfloxacin and levofloxacin) and amphynol (chloramphenicol and florfenicol), compared with human bacteria. This phenomenon may be attributable to different antibiotic selection pressures, as these antibiotics are commonly used in veterinary medicine or animal farming [36]. Amphenol has been widely used in animals but seldom (chloramphenicol) or not (florfenicol) used in human clinical practice [37]. The antibiotic susceptibility profiles of human clinical *Providencia* isolates have been reported worldwide. The isolates are generally resistant to gentamicin, tobramycin, aminopenicillins and first-generation cephalosporins, but they are commonly susceptible to carbapenems, amikacin, aztreonam, and second- and third-generation cephalosporins [38]. Similar to previous reports, the human clinical *Providencia* isolates analyzed in this study were also sensitive to aztreonam and meropenem and resistant to cephalosporins, gentamicin, aminopenicillins, and imipenem to different degrees. Until recently, few publications have reported the resistance profile of *Providencia* isolates obtained from animals or marine sources. A previous publication reported the antibacterial susceptibility of two *Providencia alcalifaciens* isolates from shark against 13 antibiotics, with the results showing that the two isolates were both sensitive to 7 antibiotics (ampicillin, amikacin, ciprofloxacin, levofloxacin, ceftazidime, imipenem and aztreonam) and that only one isolate of the two showed resistance to the other 6 antibiotics (gentamicin, streptomycin, chloramphenicol, cefalotin, amoxicillin + clavulanic acid and tetracycline) [2]. Other reports have primarily examined the treatment of animal infections caused by *Providencia* [10] or investigated the bacterial contamination of animal products, including retail meats, such as chicken, pork, and beef [39].

Notably, in this study, a larger plasmid was determined to be composed of four copies of a smaller plasmid sequence in the form of a tandem repeat in one host, which made the host to carry 5 copies of the *floR* genes. On the other hand, in *P. rettgeri* R39, the plasmids carrying the *floR* genes were not related to the intact mobile

genetic elements, although a small part of an IS97 remnant was located nearby. It has been found that the *floR* genes are related to a variety of mobile genetic elements, such as integrons, transposons and insertion sequences of the different structures that are encoded on chromosomes or plasmids across different species or genera of the bacteria [21, 40]. The homologous plasmids (> 90% identity and > 83% coverage) of pR39-8 were distributed over several bacteria of different origins, including pRCADGH-1 from a *Pasteurella multocida* isolate obtained from duck in Chengdu, Sichuan Province, China, pCCK381 of *Pasteurella multocida* and pMH1405 in *M. haemolytica*, both isolated from calves in Japan, pM3446F of *Actinobacillus pleuropneumoniae*, obtained from a pig in Greece, and the plasmid unnamed1 of an *Actinobacillus pleuropneumoniae* from a pig in South Korea, while the *P. rettgeri* R39 described in this work was isolated from a rabbit in Wenzhou, Zhejiang Province, China. These plasmids were characteristic of encoding mobilization-related genes (all carrying *mobA* and *mobC*, with the exception of pM3446F, which carried *mobA* alone) [21–23, 41, 42]. This finding might indicate that these plasmids are transferred mainly by a mobilization mechanism.

R39 carried 5 copies of the *floR* genes encoded on the two plasmids. To the best of our knowledge, no publication has documented one strain encoding more than two copies of *floR* genes. In addition, PCR screening for the *floR* gene showed that it was present not only in animal-origin *Providencia* strains but also in human-source isolates. Several studies have also reported the identification of *floR* genes in human clinical pathogens, such as *Vibrio cholera* [43] and *Klebsiella pneumonia* [44]. Florfenicol has been utilized only for animals. The spread of the *floR* gene from animal bacteria to human microbes reflects the risk to human health posed by the overuse of antibiotics in veterinary medicine or agricultural practice.

Conclusion

The results of this study indicated that the *Providencia* isolates from human clinical practice showed wider resistance spectra than those obtained from animal or freshwater fish sources, while the animal-obtained bacteria had higher antibiotic MIC levels than those did the bacteria from other sources. The *floR* gene was identified in both animal and human *Providencia* isolates. To the best of our knowledge, this report is the first to demonstrate that a so-called tandem-repeat plasmid, pR39-33, carrying four copies of the *floR* gene, is composed of four copies of another plasmid sequence in one bacterium. The mobilization genes (*mobA* and *mobC*) encoded in the resistance plasmids might play roles in the horizontal transfer of the resistance genes. These findings indicate that the widespread and heavy use of antibiotics in agricultural practice makes the emergence of high-level and multidrug-resistant bacteria increasingly serious. Dissemination of the resistance genes between animal and human bacteria may pose a severe threat to human health.

Declarations

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing Interests

The authors declare that they have no competing interests

Authors' contributions

Conceived and designed the experiments: KL, HZ, TX and QB; Performed the experiments: YJ, KZ, PZ, HL, XZ, QL, JL, XL and LL; Data analysis and interpretation: ZS, XD and JL; Drafting of manuscript: YJ, KZ, ZS and QB.

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Tables

Table 1

Bacteria and plasmids used in this work.

Strain	Characteristics	Source
<i>Providencia</i> strains	45 non-duplicated <i>Providencia</i> strains isolated from animals, seahood and patients	This study
DH5α	<i>E.coli</i> DH5α used as the recipient for resistance gene cloning	Laboratory collection
ATCC25922	<i>E.coli</i> ATCC25922, a reference strain used as control for antimicrobial susceptibility testing	Laboratory collection
pUC18/DH5α	<i>E.coli</i> DH5α carrying the cloning vector pUC18	Laboratory collection
pUC18- <i>floR</i>	pUC18 carrying the cloned <i>floR</i> gene	This study
pUC18- <i>floR</i> /DH5α	<i>E.coli</i> DH5α carrying the recombinant plasmid pUC18- <i>floR</i>	This study

Table 2

Primers used for detection of florfenicol resistance genes.

Target gene	Primer	Primer sequence (5'-3')	Amplicon size (bp)	Reference
<i>floR</i>	<i>floR-F</i> ^a	GGCTTTCGTCATTGCGTCTC	650	[26]
	<i>floR-R</i> ^a	ATCGGTAGGATGAAGGTGAGGA		
<i>fexA</i>	<i>fexA-F</i> ^a	GTA CT TGTAGGTGCAATTACGGCTGA	1,272	[24]
	<i>fexA-R</i> ^a	CGCATCTGAGTAGGACATAGCGTC		
<i>fexB</i>	<i>fexB-F</i> ^a	TTCCCACTATTGGTGAAAGGAT	816	[25]
	<i>fexB-R</i> ^a	GCAATTCCTTTTATGGACGTT		
<i>pexA</i>	<i>pexA-F</i> ^a	ACAGTGCAGGTCGAAGAACC	215	[30]
	<i>pexA-R</i> ^a	TGCATTACCAATCGACATCC		
<i>optrA</i>	<i>optrA-F</i> ^a	AGGTGGTCAGCGAACTAA	1,395	[29]
	<i>optrA-R</i> ^a	ATCAACTGTTCCCATTCA		
<i>cfr</i>	<i>cfr-F</i> ^a	TGAAGTATAAAGCAGGTTGGGAGTCA	746	[27]
	<i>cfr-R</i> ^a	ACCATATAATTGACCACAAGCAGC		
<i>estDL136</i>	<i>estDL136-F</i> ^a	TGCCCGCACCCGATTTCT	864	[31]
	<i>estDL136-R</i> ^a	GATTGGATGCACCTCGTTCTA		
<i>floR-pro</i>	<i>floR-pro-F</i> ^b	CCGGAATTCATGACCACCACACGCCCCGC	1477	-
	<i>floR-pro-R</i> ^b	CGCGGATCCTTAGACGACTGGCGACTTCT		

^a the primers for gene screening; ^b the primers for gene cloning.

Table 3

Results of the antibiotic sensitivity of the *Providencia* isolates.

Antibiotics	Patients (N=13)			Animals (N=10)			Freshwater fish (N=22)		
	S	I	R	S	I	R	S	I	R
FFC	10 (76.9%)	0 (0)	3 (23.1%)	5 (50%)	0 (0)	5 (50%)	22 (100%)	0 (0)	0 (0)
TC	0 (0)	0 (0)	13 (100%)	2 (20%)	0 (0)	8 (80%)	19 (86.4%)	0 (0)	3 (13.6%)
CHL	4 (30.8%)	2 (15.4%)	7 (53.8%)	2 (20%)	1 (10%)	7 (70%)	19 (86.4%)	3 (13.6%)	0 (0)
CTX	10 (76.9%)	0 (0)	3 (23.1%)	10 (100%)	0 (0)	0 (0)	22 (100%)	0 (0)	0 (0)
PRL	10 (76.9%)	0 (0)	3 (23.1%)	10 (100%)	0 (0)	0 (0)	22 (100%)	0 (0)	0 (0)
NOR	8 (61.5%)	0 (0)	5 (38.5%)	5 (50%)	0 (0)	5 (50%)	21 (95.5%)	0 (0)	1 (4.5%)
LVFX	6 (46.2%)	0 (0)	7 (53.8%)	3 (30%)	0 (0)	7 (70%)	11 (50%)	0 (0)	11 (50%)
IPM	1 (7.7%)	4 (30.8%)	8 (61.5%)	1 (10%)	2 (20%)	7 (70%)	18 (81.8%)	3 (13.6%)	1 (4.5%)
MEM	13 (100%)	0(0)	0 (0)	10 (100%)	0 (0)	0 (0)	22 (100%)	0 (0)	0 (0)
ATM	12 (92.3%)	1 (7.7%)	0 (0)	10 (100%)	0 (0)	0 (0)	22 (100%)	0 (0)	0 (0)
CAZ	12 (92.3%)	0 (0)	1 (7.7%)	10 (100%)	0 (0)	0 (0)	22 (100%)	0 (0)	0 (0)
AMP	7 (53.8%)	1 (7.7%)	5 (38.5%)	9 (90%)	0 (0)	1 (10%)	17 (77.3%)	2 (9.1%)	3 (13.6%)
CRO	10 (76.9%)	0 (0)	3 (23.1%)	10 (100%)	0 (0)	0 (0)	22 (100%)	0 (0)	0 (0)
KM	10 (76.9%)	0 (0)	3 (23.1%)	9 (90%)	0 (0)	1 (10%)	22 (100%)	0 (0)	0 (0)
GN	11 (84.6%)	0 (0)	2 (15.4%)	10 (100%)	0 (0)	0 (0)	22 (100%)	0 (0)	0 (0)

FFC, florfenicol; TC, tetracycline; CHL, chloramphenicol; CTX, cefotaxime; PI, piperacillin; NOR, norfloxacin; LEV, levofloxacin; IPM, imipenem; MEM, meropenem; ATM, aztreonam; CAZ, ceftazidime; AMP, ampicillin; CRO, Ceftriaxone sodium; KM, kanamycin; GN, gentamicin.

Table 4

General features of the *P. rettgeri* R39 genome.

	Chromosome	pR39-8	pR39-2.8	pR39-2.7
Size (bp)	4,502,622	8,234	2,888	2,705
GC content (%)	40.15	61.88	38.54	41.59
Predicted ORFs	4,029	8	6	3
Average ORF length (bp)	944	835	299	369
Known proteins	3479	6	2	1
Hypothetical proteins	550	2	4	2
Protein coding sequence (%)	84	81	62	41
tRNAs	78	0	0	0
rRNAs	(16S-23S-5S) * 6 (16S-23S-5S-5S) * 1	0	0	0

Table 5

The MIC results of the recombinant strain and the plasmid cured derivative (mg/L).

	FFC	CHL	AMP
R39	>512	512	2
ATCC25922	<2	8	4
DH5α	8	8	2
pUC18/DH5α	8	8	>1,024
pUC18-pro- <i>floR</i> /DH5α	256	128	>1,024
R39ΔpR39-33ΔpR39-8	4	16	2

FFC, florfenicol; CHL, chloramphenicol; AMP, ampicillin.

Figures

(a)

(b)

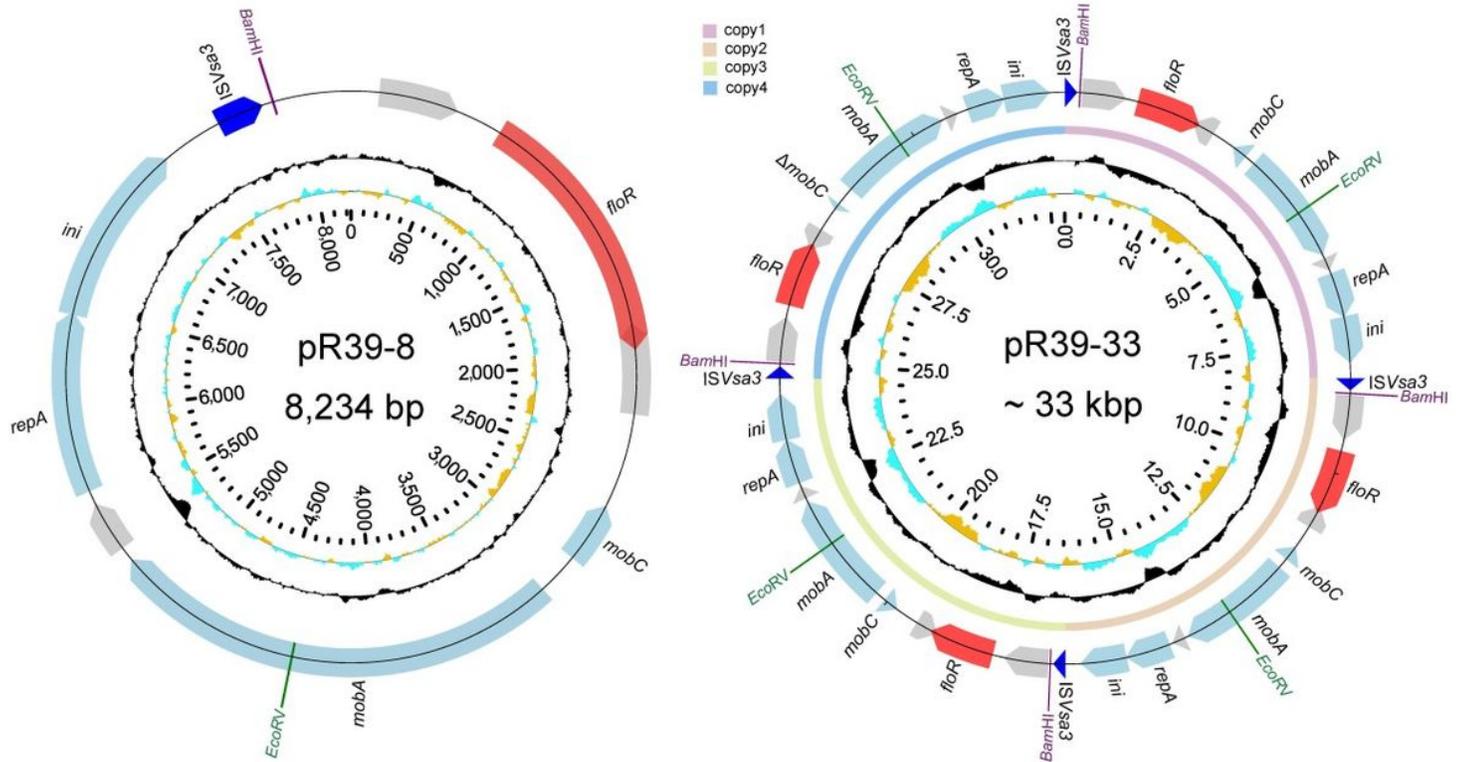


Figure 1

Circular maps of the plasmids pR39-8 and pR39-33. (a) Circular map of pR39-8 (CP066317). The three circles, counting from inside to outside, showed the position in bp, GC skew (G-C/G+C) (a positive GC skew toward the outside and a negative GC skew toward the inside) and GC content (a G+C content of more than 50% is shown toward the outside, otherwise, inward). The remaining circle shows the genes encoded, as well as EcoRV and BamHI sites on the plasmid. (b) Circular map of pR39-33 (CP066317). The three circles, counting from inside to outside, showed the position in kb, GC skew (G-C/G+C) and GC content. The remaining circles indicate that the plasmid can be divided into four parts, and each part shares the same sequence as pR39-8.

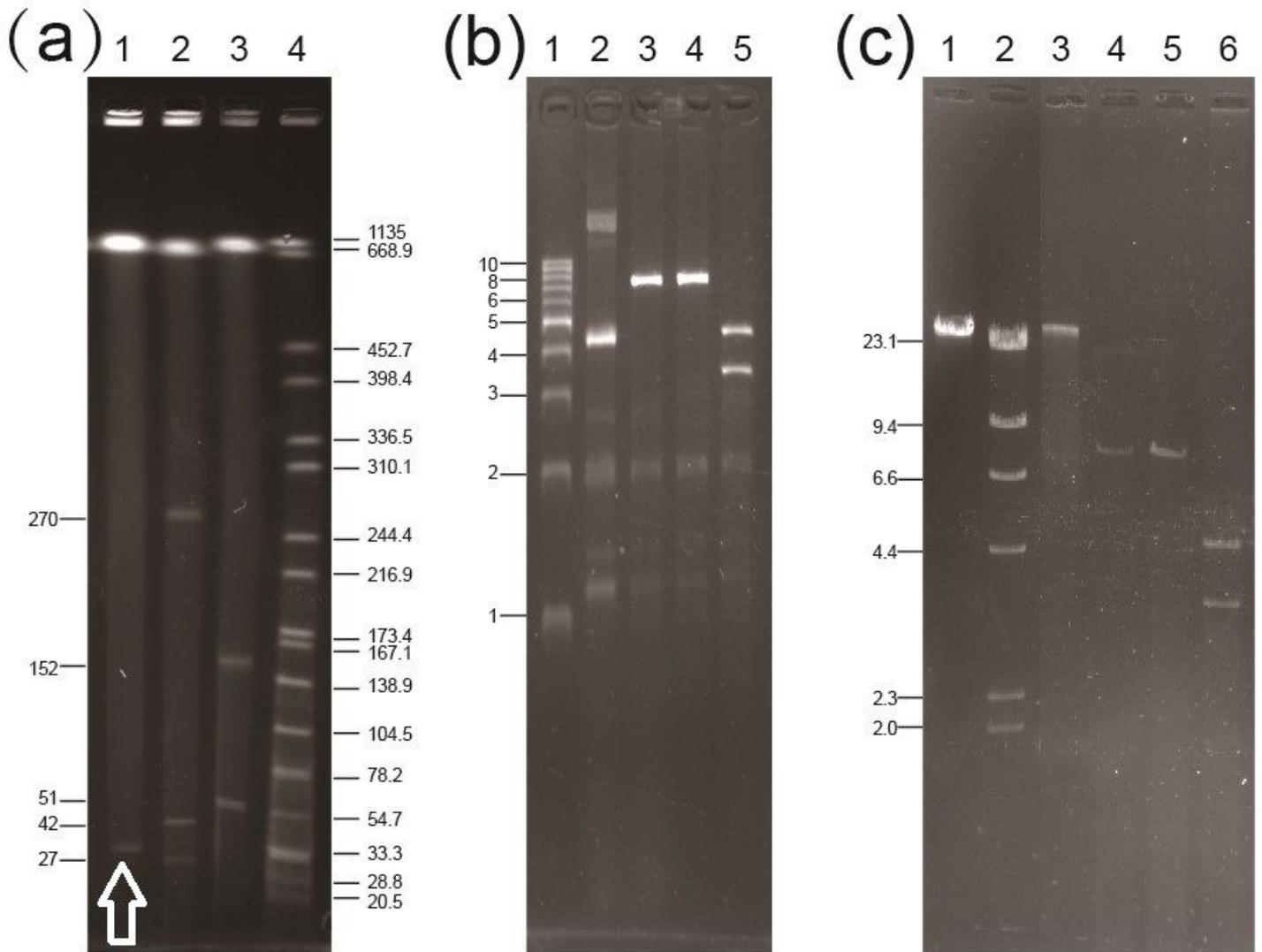


Figure 2

Electrophoresis of R39 plasmids and restriction enzyme-digested R39 plasmids (a) 1, S1-PFGE of R39 showing a plasmid band of approximately 33 kb; 2, One strain (*K. pneumoniae* R46) as a control with three known plasmids of sizes of approximately 270 kb (CP035774), 42 kb (CP035775) and 27 kb (CP035776); 3, Another strain (*Proteus cibarius* G11) as a control with two known plasmids of sizes of approximately 152 kb (CP047287) and 51 kb (CP047288); 4, *Salmonella* H9812/XbaI. (b) 1, 1 KB DNA ladder; 2, Electrophoresis of the plasmids of R39 showing pR39-33, pR39-8 plasmids and so on; 3, The plasmids of R39 digested with BamHI showing a band size of approximately 8 kb; 4, The plasmids of R39 digested with EcoRV showing a band size of approximately 8 kb; 5, The plasmids of R39 digested with BamHI+EcoRV showing two bands of approximately 3.5 and 4.5 kb, respectively. (c) 1, Lambda DNA; 2, Lambda DNA/HindIII; 3, The recovered pR39-33; 4, pR39-33/BamHI; 5, pR39-33/EcoRV; 6, pR39-33/BamHI + EcoRV.

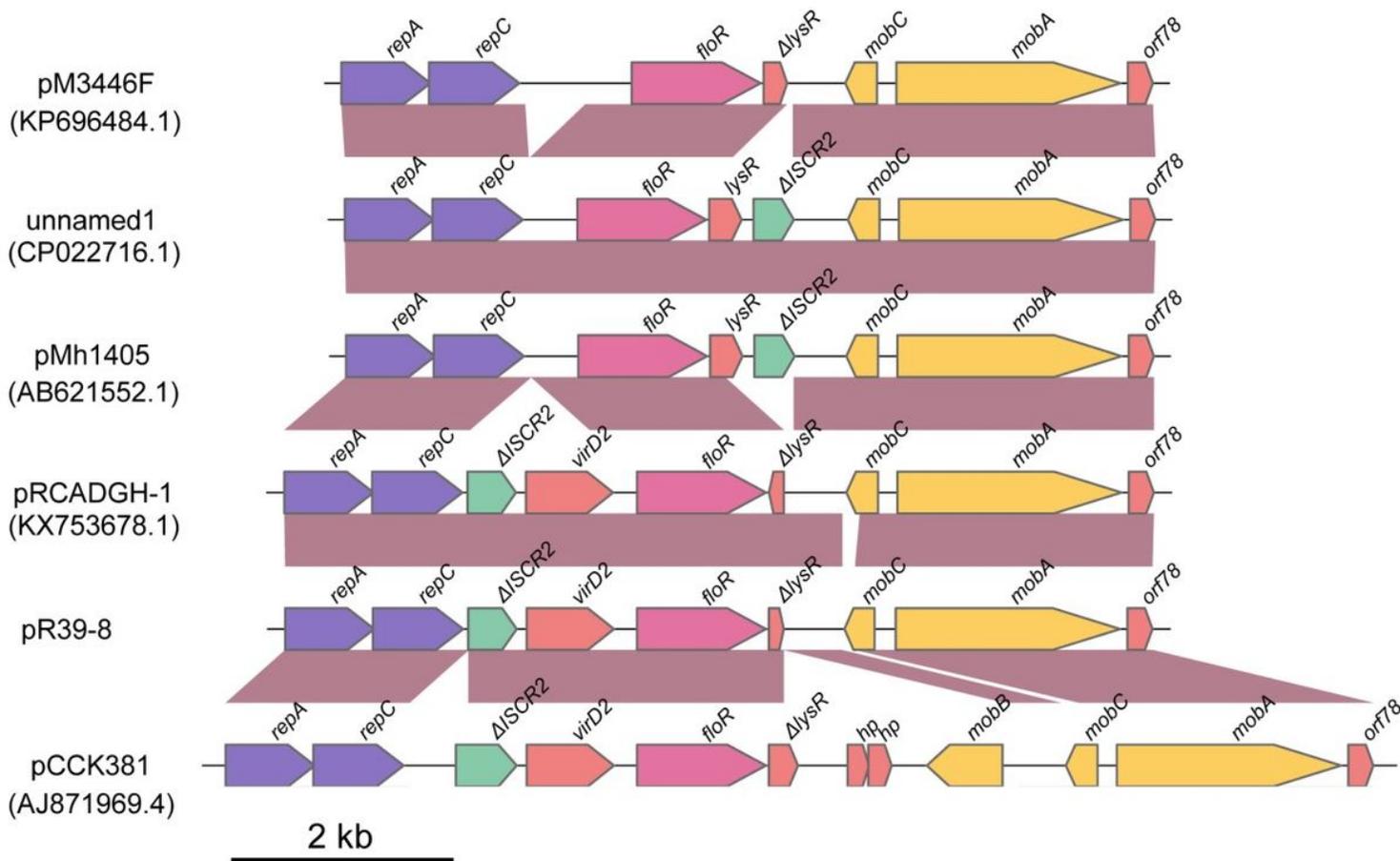


Figure 3

Comparative genomic analysis of the plasmids pM3446, unnamed1, pMh1405, pCCK381 and pR39-8. Homologous genes are marked with the same color and connected by shadow, while nonhomologous genes are left unconnected.

Supplementary Files

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