Emergence of a *floR*-carrying plasmid in extended spectrum β-lactamase (ESBL) producing *Pasteurella aerogenes*, isolated from an avian species in China

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ABSTRACT Identification and analysis of the antimicrobial resistance of *Pasteurella aerogenes* (*P. aerogenes*) isolated from poultry. For susceptibility testing in accordance with the CLSI, plasmids were extracted via alkaline lysis and transferred by $CaCl_2$ treatment. Genomic DNA of a representative *P. aerogenes* isolate was subjected to whole genome sequencing. CCCP was utilized to determine whether SF190908 contains an efflux pump. The bla_{VEB} gene was ligated with the pET-28 plasmid and transferred to *Escherichia coli* to verify it as an ESBL gene. SF190908 isolated from poultry was identified as *P. aerogenes* based upon biochemical and 16s rRNA results. The isolate showed high MIC values for eight antimicrobials. Sequencing results showed that the mobile element-mediated antimicrobial resistance gene cluster conferred antimicrobial resistance on the strain, and a single 5,105-bp plasmid, designated pRCAD0752PA-1, was isolated. Four antimicrobial resistance gene clusters were identified in the SF190908 chromosome; one antimicrobial resistance gene cluster carried the bla_{VEB} gene, which was verified as ESBL according to the CLSI and was detected in *Pasteurellaceae* for the first time, to the best of our knowledge. The efflux pump may confer antimicrobial resistance to SF190908. *P. aerogenes* isolated from poultry showed resistance genes encoded in mobile elements that confer multi-antimicrobial resistance to SF190908. The antimicrobial-resistant plasmid pRCAD0752PA-1 was isolated in SF190908 and conferred resistance to florfenicol. This study indicates an urgent need to increase efforts to monitor the spread of *P. aerogenes* multi-antimicrobial-resistant strains and plasmids, especially in newly discovered at-risk species such as poultry.

Key words: bla_{VEB}, *floR*, plasmid-mediated resistance, *Pasteurella aerogenes*, multi-antimicrobial resistance

2022 Poultry Science 101:102207
https://doi.org/10.1016/j.psj.2022.102207

INTRODUCTION

Pasteurella aerogenes (*P. aerogenes*) is an important opportunistic pathogen that induces abortion in pigs (Hommez and Devriese, 1976), cattle, cats and rabbits (Thigpen et al., 1978). It was also isolated from a stillborn child and mother (Thorsen et al., 1994). *P. aerogenes* reportedly causes vertebral osteomyelitis in humans with normal immunity (Quiles et al., 2000). *P. aerogenes* has been isolated from girls with neurogenic bladder and ulcers or wounds in humans (Ejlertsen et al., 1996; Alaygut and Engin, 2018). To the best of the authors' knowledge, this is the first report of *P. aerogenes* isolated from an avian species.

There are a few reports of resistant strains of *P. aerogenes*. Kehrenberg and Schwarz (Kehrenberg and Schwarz, 2000, 2001, 2011) reported that the plasmid pPAT1, pPAT2 carries the tetracycline resistance gene and the plasmid pCCK343 carries the trimethoprim and aminoglycoside antimicrobial resistance gene cluster in *P. aerogenes*. Livrelli et al. (1988) reported a strain of *P. aerogenes* containing the bla_{ROB-1} β-lactamase gene in the chromosome.

The production of extended spectrum β-lactamases (**ESBL**s) is an important cause of β-lactam antibiotic resistance. ESBLs are β-lactamases that can inactivate broad- and narrow-spectrum cephalosporins and monocyclic

Received June 10, 2022.
Accepted September 7, 2022.

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antibiotics. bla_{VEB} is an atypical small ESBL resistance gene (Espinal et al., 2017), which was first found in *Pseudomonas aeruginosa* in Vietnam, and subsequently found in large numbers in *Enterobacter* species and *P. aeruginosa* in Southeast Asia (Maurya et al., 2014). To the best of our knowledge, there is no report of the bla_{VEB} gene in *Pasteurellaceae*.

Florfenicol is an animal-specific antibiotic used in veterinary clinics to treat bacterial diseases (Graham et al., 1988). The *floR* gene can encode an efflux pump protein that specifically effluxes florfenicol, thereby conferring florfenicol resistance to bacteria (Wu et al., 2006). Currently, *Haemophilus parasuis* (Li et al., 2015), *Pasteurella multocida* (Zhu et al., 2020) and *Actinobacillus pleuropneumoniae* (da Silva et al., 2017) have been reported as having plasmids carrying the *floR* gene, but there is no report that *P. aerogenes* carries the *floR* gene plasmid.

The aim of the present study was to analyze in detail the genetic environment of the antimicrobial resistance genes of the first strain of *P. aerogenes* isolated from ducks and the transfer ability of *floR*-carrying plasmids.

METERIALS AND METHODS

Bacteria Isolation and Identification

On a large-scale duck farm in Sichuan, China, in 2019, a healthy duck throat swab sample was inoculated on blood tryptic soy agar (**TSA**) plates and cultured at 37°C for 24 h. The dominant single colony was selected for purification. The colonies were biochemically identified using biochemical reaction tubes. Then, the strains were identified by polymerase chain reaction (**PCR**) using the 16S rRNA gene universal primers, sequencing and BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) comparison.

MIC Measurements

The MICs of antimicrobials against original isolates were investigated, and transformants were determined by broth microdilution according to the standard of CLSI (CLSI, 2018).

The medium for measuring the MIC of *E. coli* is Mueller-Hinton broth (**MH**) and the medium for measuring the MIC of *P. aerogenes* is cation-adjusted Mueller-Hinton broth (**CAMHB**).

Dilute the concentration of the antimicrobial to 1,024 mg/L−0.5 mg/L using the corresponding medium. The log phase bacterial solution was diluted to $OD_{600} = 0.04$ and added to the microplates in equal proportion with antimicrobials. The inoculated microplates were incubated at 37°C for 24 h.

Whole-Genome Sequencing

High-quality genomic DNA was extracted with a TIANamp Bacteria DNA Kit (Tiangen Biotech Co., Ltd., Beijing, China). Sequencing of *P. aerogenes* SF190908 was conducted using an Illumina HiSeq 2500 and assembly was performed with Spades version 3.14.0, incorporating different k-mer sizes according to the read lengths while taking care to reduce assembly errors; contigs below 200 base pairs were excluded.

Transformation of pRCAD0752PA-1

To explore the resistance level mediated by the resistance gene for pRCAD0752PA-1, Plasmid pRCAD0752PA-1 was transformed into *E. coli* DH5α by $CaCl_2$. Transformants were selected using Luria −Bertani agar (**LB**) plates containing 32 μg/mL florfenicol. The transformant was verified by PCR. floR -F (5'-CTACCAGTCGTCGGTTAGACGAC-3') and floR -R (5'- GATTCGTCATGACCACCAC -3').

Expression of bla_{VEB} Genes in E. coli

To confirm the function of bla_{VEB} in β-lactam resistance, bla_{VEB} was amplified with the primers pET28a-bla_{VEB} -F (5'-CCCAAGCTTCCTATGAGCCAGTGTTAGC-3') and pET28a- bla_{VEB} -R (5'-CGGAATTCCCAGATAGGAGTACAGAC-3'). The amplicon was linked to pET28a by restriction enzyme ligation. The new plasmids were transferred to DH5α by $CaCl_2$ and coated on LB medium containing 40 μg/mL kanamycin. The transformant was verified by PCR. The ESBL function of positive transformants was identified according to the standard of CLSI.

Efflux Pump Determination

Since the efflux pump inhibitor carbonyl cyanide m-chlorophenylhydrazone (**CCCP**) has an inhibitory effect on the growth of *P. aerogenes*, when determining the MIC of *P. aerogenes* to antimicrobials after adding CCCP, it is necessary to add a second inhibitory concentration (1/2 inhibitory concentration) of CCCP to the diluted bacterial solution (final concentration, 1.5 mg/L). The final judgment result is that when the MIC value of the antimicrobials is reduced to at least one-fourth of the original, it can be judged as positive for the active efflux pump (Valdezate et al., 2002).

RESULTS AND DISCUSSION

The isolate formed a greyish-white circular colony on the blood TSA plate and a smooth, moist transparent dew-like colony on the ordinary plate. The results of biochemical identification showed that decomposition of glucose, galactose, sucrose, fructose, maltose, and arabinose occurred, whereas there was no decomposition of rhamnose, sorbitol, or mannitol. The catalase and oxidase tests were negative whereas the urease test was positive. These findings indicated similar biochemical characteristics as those of the reported *P. aerogenes* (Hommez and Devriese, 1976; Lester et al., 1993).

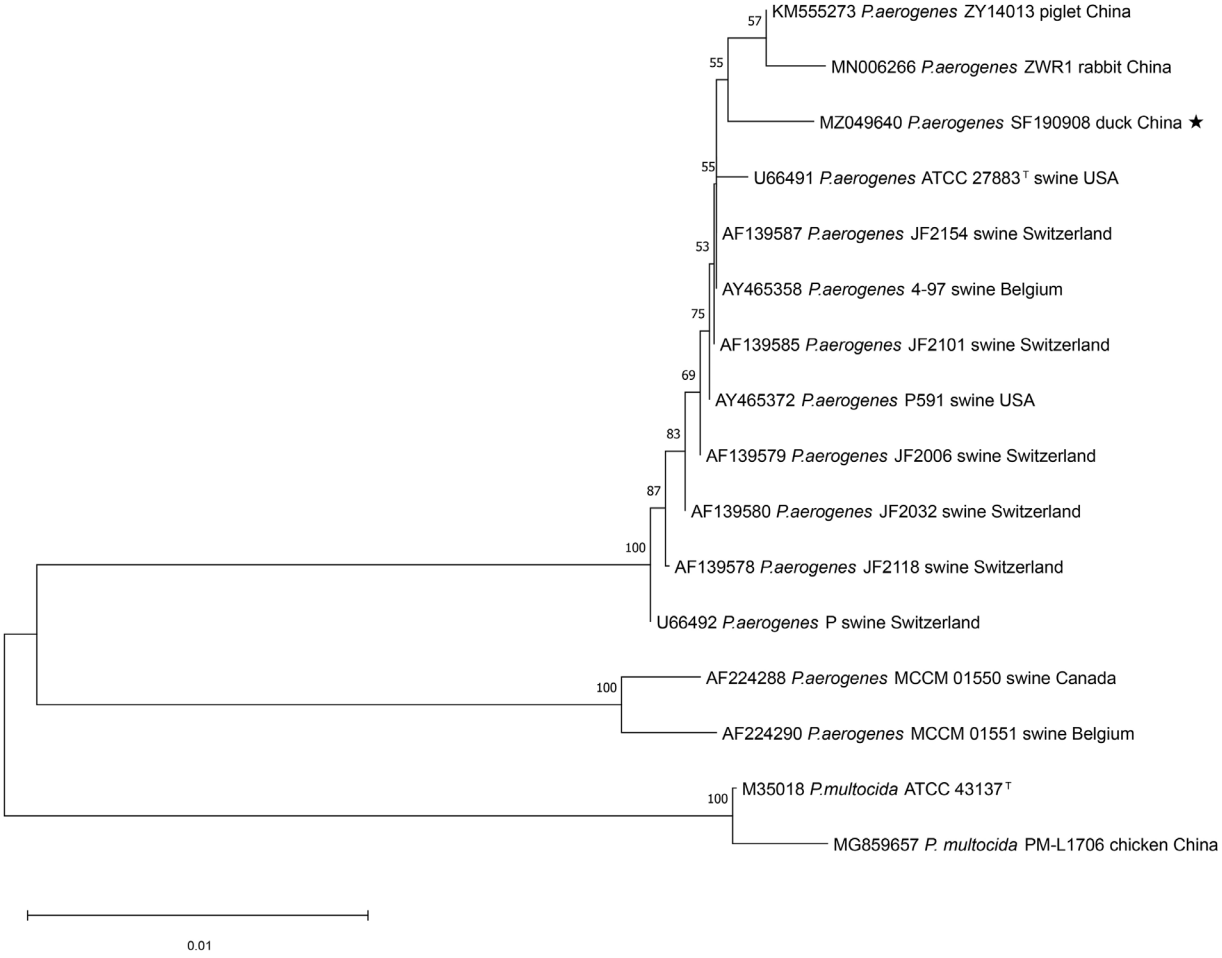

Figure 1. Phylogenetic relationships among strains of Pasteurellaceae. June., members of existing species of *P. aerogenes* and related members of the family Pasteurellaceae based on neighbour-joining analysis of nearly full-length 16S rRNA gene sequences. Each strain contains a GenBank accession number, host, and location of separation. Bar, 0.01 substitutions per nucleotide position.

The 16S rRNA gene sequence similarity between SF190908 and 13 *P. aerogenes* isolates in 13 NCBI ranges from 93.98 to 99.93% (Figure 1). Phylogenetic analysis classified SF190908 and 2 *P. aerogenes* isolated in China into one cluster (Tamura et al., 2021). The sequence

P. aerogenes SF190908 exhibited resistance to as many as 8 types of antimicrobials (Table 1). SF190908 antimicrobial resistance included not only rifampin (8 mg/L), erythromycin (32 mg/L), ofloxacin (128 mg/L), tetracycline (128 mg/L), penicillin (256 mg/L), chloramphenicol (256 mg/L), florfenicol (256 mg/L), and ampicillin (512 mg/L) but also to streptomycin, kanamycin, cefotaxime sodium, cefradine, aztreonam, trimethoprim, and sulfamethoxazole at doses greater than 512 mg/L.

Based on SF190908 genome sequence analysis, 4 antimicrobial-resistant gene clusters and an antimicrobial-resistant plasmid were identified in SF190908. The plasmid, named pRCAD0752PA-1, is 5,105 bp in length and comprises the florfenicol resistance gene *floR* encoding a major facilitator superfamily (MFS) efflux pump, the transcriptional regulator *lysR* and the replication gene *rep* (Figure 2). The FloR protein has 99.75% identity and 100% coverage with *Escherichia coli* FloR (GenBank accession no. WP_072679283.1).

GenBank database searches identified three plasmids that showed high sequence homology with pRCAD0752PA-1,

Table 1. MIC value of strains with or without CCCP.

	MIC(mg/L)							
Strain	Ampicillin	Penicillin	Cefotaxime Sodium	Cefradine	Aztreonam	Chloramphenicol	Florfenicol	Erythromycin
SF190908	512	256	>512	>512	>512	256	256	32
SF190908 + CCCP	512	256	>512	>512	>512	256	256	8
	MIC(mg/L)							
Strain	Kanamycin	Streptomycin	Ofloxacin	Tetracycline	Polymyxin	Sulfamethoxazole	Trimethoprim	Rifampin
SF190908	>512	>512	128	128	4	>512	>512	8
SF190908 + CCCP	>512	>512	8	64	2	>512	>512	8

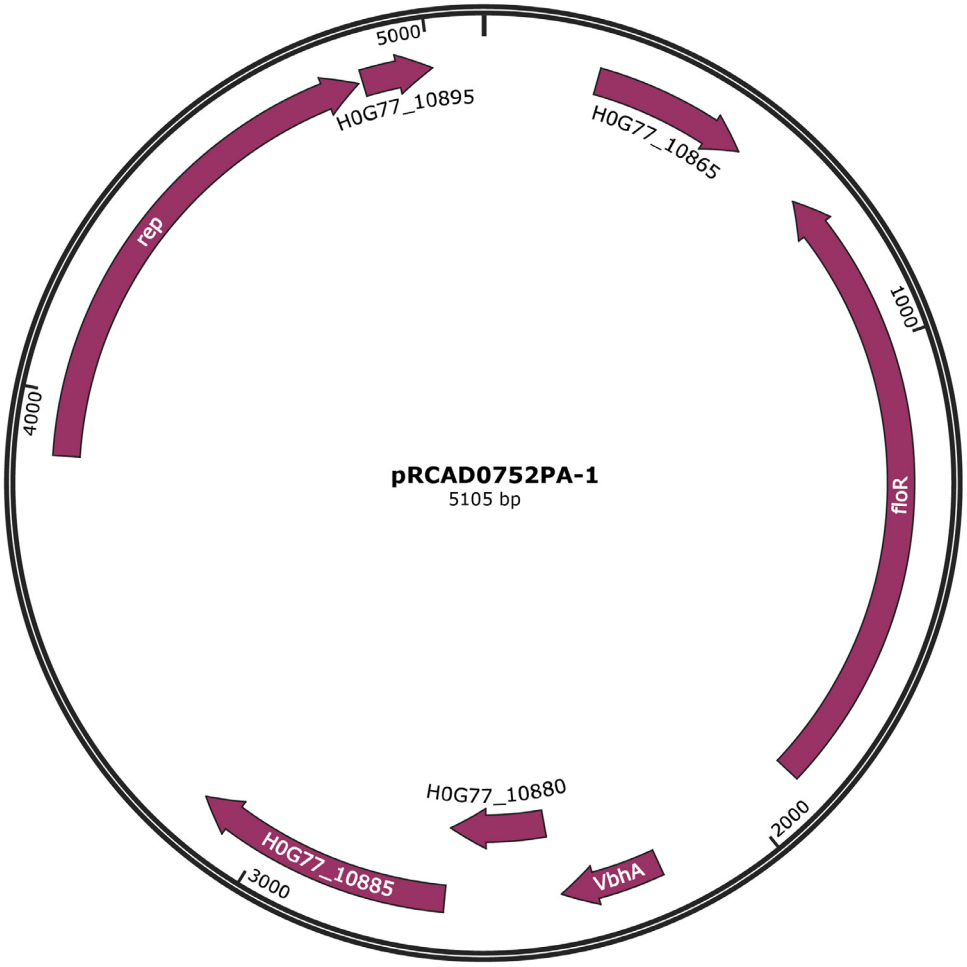

Figure 2. Plasmid profile of pRCAD0752PA-1 from *P. aerogenes* strain SF190908.

including the 5,279 bp flor-carrying plasmid pHPSGC of *Glaesserella parasuis* isolated from sick pig lungs in China (GenBank accession no. KX966395.1), the 4,597 bp *tet*(B)-carrying plasmid pHPS1019 of *G. parasuis* in China (GenBank accession no. HQ622101.1), the 92,076 bp carrying the multiple antimicrobial resistance gene plasmid pCP61-IncFIB of *E. coli* isolated from a pig in China (GenBank accession no. NZ_CP053729.1).

Among the three similar plasmids, pHPSGC has the highest nucleotide identity and coverage, which has 98% nucleotide coverage and 98.58% identity to plasmid pRCAD0752PA-1 (Figure 3). pRCAD0752PA-1, which has 55% nucleotide coverage and 99.82% identity to pHPS1019, contains the Rep protein. In particular, the rest of pRCAD0752PA-1 has 98% nucleotide coverage and 98.52% identity to pCP61-IncFIB and contains the LysR protein and FloR protein. We suspect that the source of pRCAD0752PA-1 may be related to the recombination of pHPS1019 and pCP61-IncFIB plasmids.

Plasmid pRCAD0752PA-1 transfer was performed by $CaCl_2$ treatment into the *E. coli* DH5α. The MIC of florfenicol in transformants compared with the corresponding 'empty' recipient strains increased by a factor of 32-

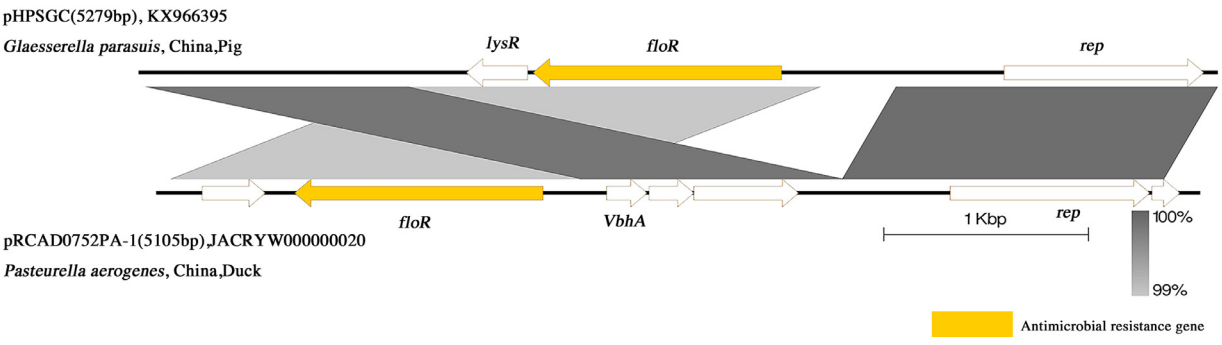

Figure 3. The plasmid pRCAD0752PA-1 was compared with the plasmid pCP61-IncFIB from *G. parasuis*. Grey areas represent similar sequences.

fold (from 8 mg/L to 256 mg/L). These results indicate that the resistance of strain SF190908 to florfenicol was derived from this small plasmid.

SF190908 antimicrobial-resistant gene cluster 1 is 9,244 bp in size and has two IS3 family transposase at the beginning and end of the gene cluster. Upstream of antimicrobial-resistant gene cluster 1, two replication genes, *rep*C and *rep*A, and 2 mobilization genes, *mob*A/L and *mob*C, were identified. This region has 98.21% nucleic acid homology identity and 85% nucleic acid coverage to plasmid pIE1115 (GenBank accession number AJ293027.1). The rest of the antimicrobial-resistant gene cluster 1 harbored 4 consecutive resistance genes. The first gene is 438 bp in size and is characterized by a truncated chloramphenicol efflux pump *cmlA*. Compared with the reported *cmlA* gene, the *cmlA* gene generally has a 30 to 40 bp deletion at the 3′end, and the second gene is 453 bp in size and contains the gene *ARR-2* encoding NAD(+)-rifampin ADP-ribosyltransferase, which confers resistance to rifampin antibiotics. The third gene is 534 bp in size and contains the gene *aadB* encoding aminoglycoside nucleotidyltransferase, which confers resistance to aminoglycoside antibiotics. The fourth gene is 900 bp in size and contains the gene bla_{VEB} encoding for an ESBL of 300 amino acids, which confers resistance to most β-lactam antibiotics. Database searches identified truncated *cmlA, ARR-2, aadB*, and bla_{VEB} genes indistinguishable from those of *P. aerogenes* SF190908, mainly in the *P. multocida* strain RCAD0259 plasmid pRCADGH-2 (GenBank accession number KX753679.1). The upper and lower parts of antimicrobial-resistant gene cluster 1 have high homology identity and coverage with plasmid pIE1115 and plasmid pRCADGH-2, respectively, which indicates that plasmid pIE1115 and plasmid pRCADGH-2 may have recombined and integrated into the chromosome of strain *P. aerogenes* SF190908 (Figure 4).

At present, there has not been any report about the bla_{VEB} gene in *Pasteurellaceae*. In this study, we reported the first bla_{VEB} gene in *Pasteurellaceae* and verified its function. The bla_{VEB} gene was amplified by PCR and ligated to the pET-28 plasmid, and the pET-28 plasmid ligated with the bla_{VEB} gene passed calcium is transferred into *E. coli* DH5α.

Compared with the original *E. coli* DH5α, the transformant *E. coli* DH5α has a 32-fold increase in the MIC value of ampicillin (from 8 mg/L to 256 mg/L), a 64-fold increase in the MIC value of ceftazidime (from 4 mg/L to 512 mg/L), and a 128-fold increase in the MIC value of cefotaxime (from 4 mg/L to 512 mg/L).

The phenotype of ESBL of the DH5α transformant was determined according to CLSI regulations. The results of antimicrobial susceptibility testing showed that the diameter of the inhibitory zone of the transformant to cefotaxime (30 μg) was 16 mm; The diameter of the zone of inhibition for cefotaxime/clavulanic acid (30/10 μg) was 29 mm; the diameter of the zone of inhibition for ceftazidime (30 μg) was 8 mm; and the diameter of the zone of inhibition for ceftazidime/clavulanic acid (30/10 μg) was 25 mm.

These results satisfy the criterion that the diameter of the inhibitory ring after clavulanic acid is added to any one of the two third-generation cephalosporins is increased by 5 mm compared with that without clavulanic acid. The bla_{VEB} gene was confirmed to encode ESBL and endow *P. aerogenes* with resistance to cephalosporin antibiotics.

SF190908 antimicrobial-resistant gene cluster 2 is 11,574 bp in size and contains 12 open reading frames (ORFs), and an *IntI2* gene encoding type 2 integrase was found at the beginning of antimicrobial-resistant gene cluster 2. The integron carries 3 antimicrobial-resistant gene cassettes, and a transposase of the IS1 family was found downstream of the antimicrobial-resistant gene

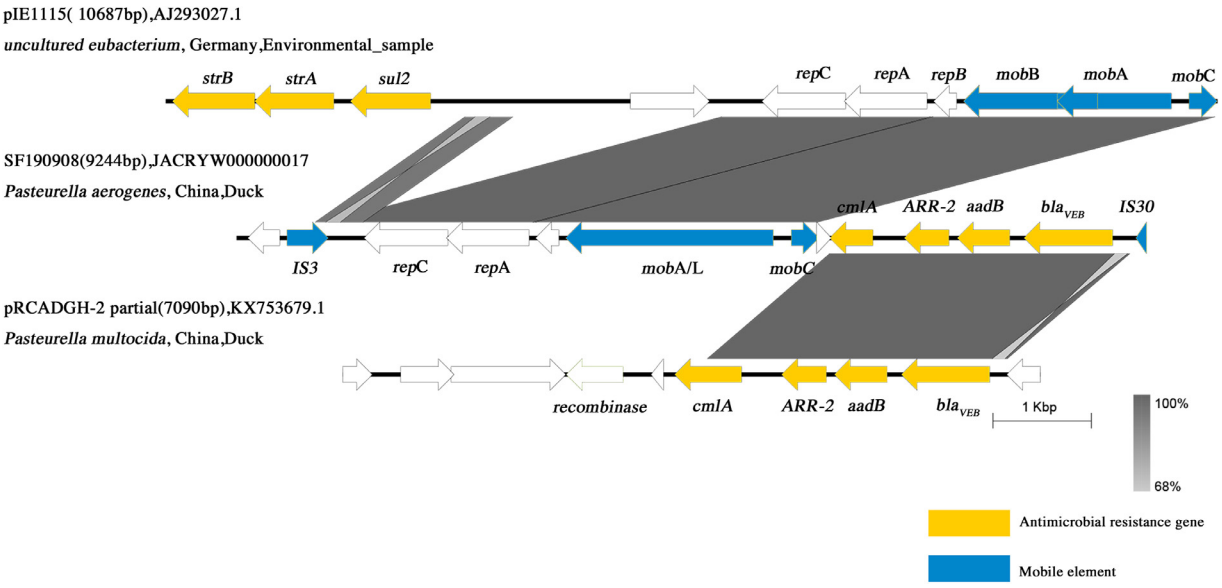

Figure 4. SF190908 resistance gene cluster 1 was compared with the plasmid pRCADGH-2 from *P. multocida* and the plasmid pIE1115 from *uncultured Eubacterium*. Grey areas represent similar sequences.

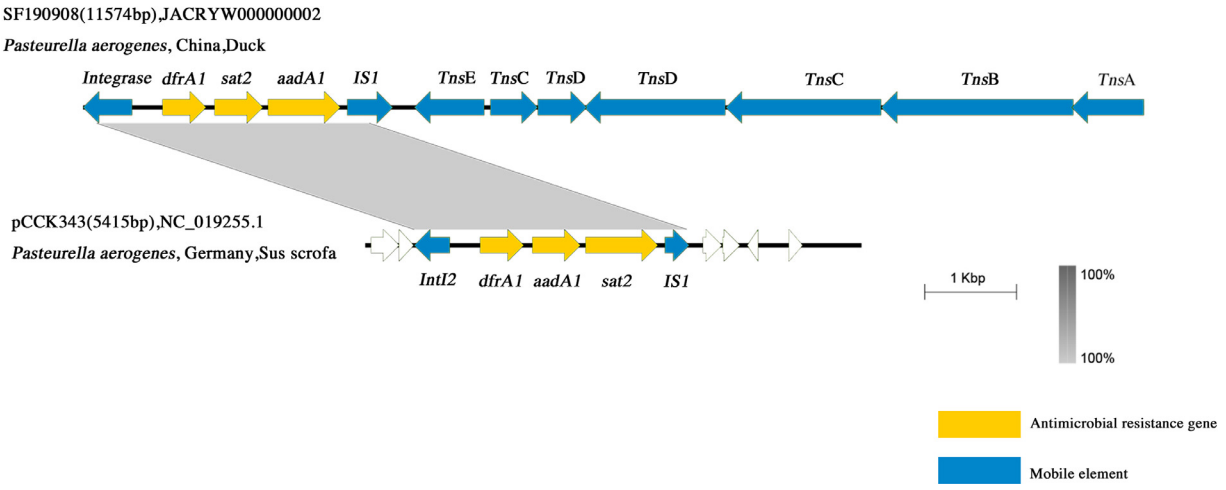

Figure 5. SF190908 resistance gene cluster 2 was compared with the plasmid pCCK343 from *P. aerogenes*. Grey areas represent similar sequences.

cassette. The first gene cassette is 474 bp in size and contains the *dfrA1* gene, which encodes the dihydrofolate reductase to confer resistance to trimethoprim. The second gene cassette is 525 bp in size, contains the *sat* gene, encodes streptomycin acetyltransferase, and confers resistance to streptomycin. The third gene cassette is 789 bp in size and contains the *aadA1* gene, encoding aminoglycoside nucleotide transferase, which confers resistance to aminoglycoside antibiotics. This region has 99.9% nucleic acid homology and 55% nucleic acid coverage with the plasmid pCCK343 of *P. aerogenes* isolated in Germany (GenBank accession number FR687372.1) (Kehrenberg and Schwarz, 2011) (Figure 5). The rest of the antimicrobial-resistant gene cluster 2 contains eight ORFs, including *Tns*E, *Tns*C, *Tns*D, *Tns*B, and *Tns*A genes, which mainly encode Tns transposase.

The size of SF190908 resistance gene cluster 3 is 3,899 bp and contains six ORFs. It contains the efflux pump gene *tet*(B) encoding the MFS family, which confers bacterial resistance to tetracycline. There is a transcriptional regulator *tet*(R) upstream of the *tet*(B) gene. SF190908 resistance gene cluster 3 has 99.8% nucleic acid homology and 89% nucleic acid coverage with previously reported *P. multocida* GH161213 (Zhu et al., 2020) (Figure 6). Secondly, it has 99.75% nucleic acid similarity with the region containing *tet*(B) and *tet*(R) genes in the plasmid pPAT2 isolated from *P. aerogenes* (Kehrenberg and Schwarz, 2001). The rest of the plasmid sequence is not shown. (GenBank accession number AJ278685.1).

SF190908 antimicrobial-resistant gene cluster 4 is 7,169 bp in size and comprises a total of 5 different resistance genes as well as one regulatory gene. The first gene is 189 bp in size and encodes a putative transcriptional regulator. The second gene is 816 bp in size and encodes sulfonamide-resistant dihydropteroate synthase sul2, achieving combined resistance to sulfamethoxazole. The third gene is 642 bp in size and encodes type A3 chloramphenicol O-acetyltransferase catA3, achieving combined resistance to chloramphenicol. The following three genes are 804 bp, 837 bp, and 816 bp in size, respectively encoding aminoglycoside O-phosphotransferase strA, aminoglycoside O-phosphotransferase strB, and aminoglycoside O-phosphotransferase aph(3′)-Ia, achieving combined resistance to aminoglycoside antibiotics.

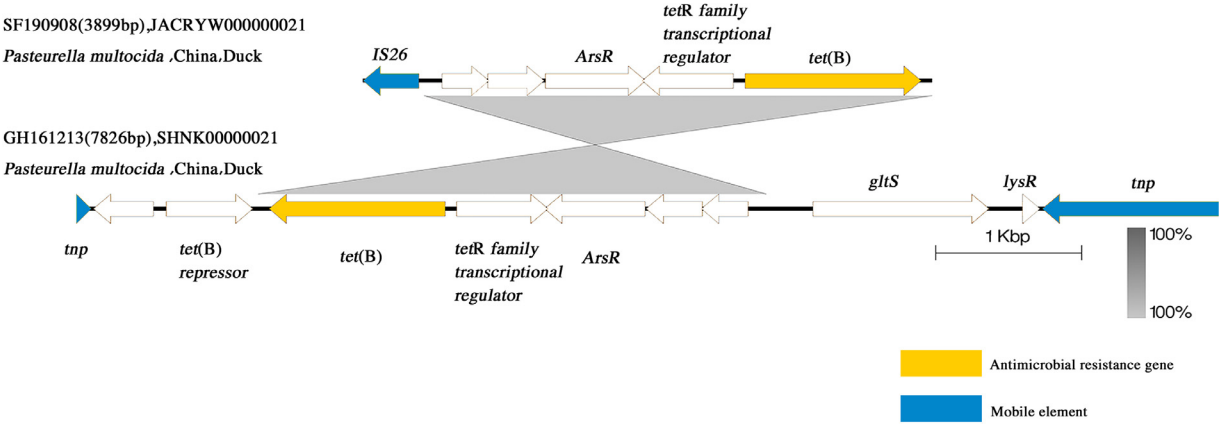

Figure 6. Comparison of SF190908 resistance gene cluster 3 with resistance gene cluster from *P. multocida* GH161213. Grey areas represent similar sequences.

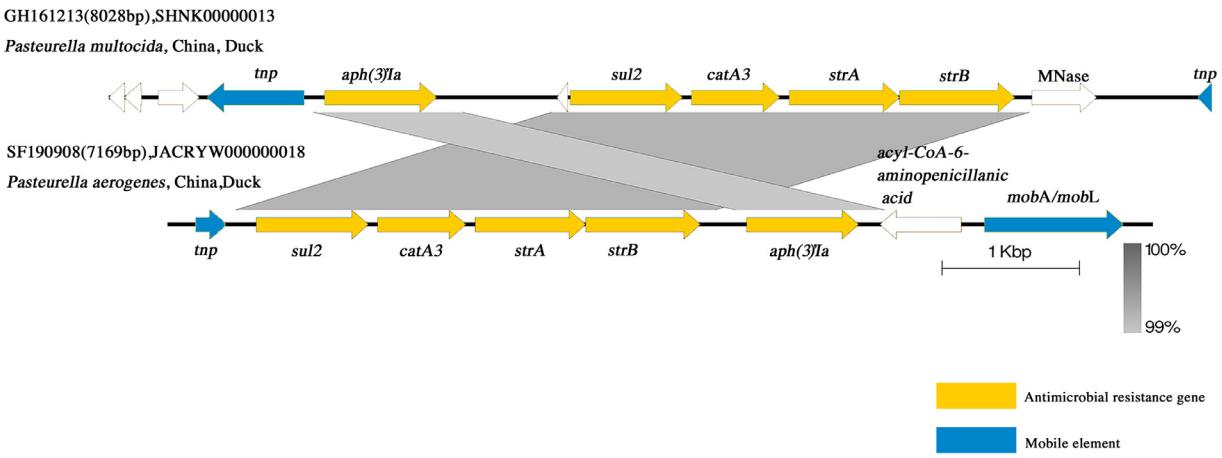

Figure 7. Comparison of SF190908 resistance gene cluster 3 with resistance gene cluster from *P. multocida* GH161213. Grey areas represent similar sequences.

Antimicrobial resistance genes have 100% amino acid coverage and not lower than 98.15% identity to the *P. multocida* GH161213 (GenBank accession number SHNK00000013) (Figure 7).

Plasmid pIE1115 was identified as not only associated with the resistance gene cluster 1 of SF190908, but also with the resistance gene cluster 4 of SF190908. pIE1115 was compared with two antimicrobial resistance gene clusters of SF190908, and the nucleic acid has a coverage of 81% and an identity of not less than 98%, which contains *rep*C, *rep*A, *mob*A/L, *mob*C, *strA, strB*, and *sul2* genes. This phenomenon suggests that the source of SF190908 resistance may be derived from plasmid pIE1115.

After adding CCCP to SF190908, comparative testing revealed distinctly lower MIC values of some antimicrobials in the SF190908 with added CCCP compared with SF190908 without CCCP added (Table 1).

The MIC value of 2 types of antimicrobials, ofloxacin and erythromycin, showed a difference of 4 times or more in SF190908. We speculated that the reason for this phenomenon is that SF190908 has efflux pump resistance.

Sequencing analysis of the genomes of SF190908 indicated that they encode several efflux pump systems. One of the efflux systems involved in this resistance phenotype is the AcrAB multidrug efflux system, which is a resistance-nodulation-cell division family efflux pump capable of multidrug efflux, which is composed of the cytoplasmic membrane protein AcrB, the periplasmic protein AcrA and the repressor AcrR, which can encode AcrAB. They encode homologs of an efflux pump with 65.74% to 74.95% amino acid identity to AcrAB of *Haemophilus influenzae*.

The AcrAB efflux pump is involved in resistance to multiple antimicrobials, as proven in studies by Padilla et al. (2010), who demonstrated that the AcrAB efflux pump in *Klebsiella pneumoniae* is involved in the resistance of polymyxin B, quinolones, erythromycin, tetracycline, and β-lactam antimicrobials.

In addition, another efflux pump, MacAB-Tolc, was found in the genomes of SF190908. The MacAB-Tolc efflux pump system is an ATP-binding cassette (ABC) family efflux pump capable of erythromycin efflux (Kobayashi et al., 2001). They encode homologs of a tripartite efflux pump with 82.17% to 92.98% amino acid identity to MacAB-Tolc of *Actinobacillus seminis*.

In conclusion, we report the emergence of multi-antimicrobial-resistant strains of *P. aerogenes*, which were isolated from poultry for the first time. Multiple antimicrobial resistance gene clusters were identified in the chromosomes of SF190908. One of the antimicrobial-resistant gene cluster contains the ESBL bla_{VEB} gene, which was identified for the first time in *Pasteurellaceae*. Simultaneously, a plasmid containing the *floR* gene, capable of conferring florfenicol resistance, appeared in SF190908. The long-term existence of multi-antimicrobial-resistant attenuated *P. aerogenes* in the environment facilitates the horizontal transfer of resistance genes. This research suggests that we should use antibiotics rationally in production practice. Furthermore, there is an urgent need to increase efforts to monitor the spread of *P. aerogenes* multi-antimicrobial-resistant strains and plasmids, especially in newly discovered at-risk species such as poultry, for which monitoring has not yet been recognized as a need, and thus methods of prevention and control of viral spread have yet to be developed.

ACKNOWLEDGMENTS

This work was supported by Sichuan Veterinary Medicine and Drug Innovation Group of China Agricultural Research System (SCCXTD-2021-18), China Agriculture Research System of MOF and MARA.

Availability of data: The data supporting the results of this paper is available in the NCBI Whole-Genome Sequencing database. The whole genome sequence of *P. aerogenes* strain SF190908 and plasmid pRCAD0752PA-1 have been deposited in GenBank under accession numbers JACRYW000000000 and JACRYW000000020, respectively. Submitted BioProject accession number is PRJNA645766.

DISCLOSURES

The authors declare no competing interests.

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