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Identification and pathogenicity of an XDR *Streptococcus suis* isolate that harbours the phenicol-oxazolidinone resistance genes *optrA* and *cfr*, and the bacitracin resistance locus *bcrABDR*

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ABSTRACT

One hundred and seven *Streptococcus suis* isolates were collected from healthy pigs or asymptomatic carriers in Jiangsu, China in 2016–2017. Thirty-eight percent of the isolates were linezolid-resistant and all carried the *optrA* gene. Among them, one isolate, SFJ44, was resistant to all 20 of the antibiotics tested, except for ceftiofur, and thus exhibited an extensively-drug-resistant phenotype. This isolate carried the *optrA* gene and the bacitracin resistance locus *bcrABDR* on an antibiotic-resistance-associated genomic island (ARGI1), and harboured the resistance genes *cfr, aadE, sat4, spw*-like, *aphA3, mef*(A), *msr*(D), *erm*(A)-like, *erm*(B), *tetAB*(P)', *tet*(M) and *catQ* on ARGI2∼4. The IS*1216E-bcrABDR*-IS*Enfa1* segment showed >99.9% sequence identity to corresponding sequences from other species. The *cfr* gene was located on ARGI4, and two IS*6* family insertion sequences, IS*1216E* and IS*Teha2*, were found upstream and downstream of *cfr*-ΔIS*Enfa5*, respectively. A circular intermediate of *bcrABDR*-IS*Enfa1* was detected, suggesting the role of IS*Enfa1* in dissemination of *bcrABDR*. Other antibiotic resistance genes might be acquired from different Gram-positive pathogens. Infection of zebrafish showed that SFJ44 exhibited a virulence level comparable to serotype 2 hypervirulent strain SC070731, highlighting the need for surveillance of the pathogenicity of multi-drug-resistant *S. suis* isolates. This is the first report of the co-existence of *optrA* and *cfr*, and of the *bcrABDR* locus in streptococci. As it has been suggested that *S. suis* may act as an antibiotic resistance reservoir contributing to the spread of resistance genes to major streptococcal pathogens, the potential dissemination of these resistance genes among Gram-positive bacteria is of concern and routine surveillance should be strengthened.

1. Introduction

Oxazolidinones, including linezolid and tedizolid, show high efficiency against most Gram-positive pathogens [1]. However, oxazolidinone resistance in Gram-positive bacteria has been reported increasingly in recent years. The first transferable oxazolidinone resistance gene, *cfr*, which mediates the transfer of resistance to linezolid as well as phenicols, lincosamides, pleuromutilins and streptogramin A [2], has been detected in a variety of Gram-positive and -negative bacteria [3–6]. The *cfr*-like genes, *cfr*(B) and *cfr*(C), have been discovered in *Clostridium difficile, Enterococcus faecium* and *Campylobacter* spp. [7–9]. Recently, a second transferable oxazolidinone resistance gene, *optrA*, which confers resistance to phenicols and oxazolidinones (PhO), has been reported in enterococci in China [10]. *optrA* has also been detected in other countries and in streptococci [11–13]. A novel oxazolidinone resistance gene, *poxtA*, has been reported recently [14]. In streptococci, a single report of oxazolidinone resistance conferred by *cfr* was characterized in *Streptococcus suis* in 2013 [15]. More recently, *optrA*-mediated PhO resistance was reported in streptococci, again in *S. suis,* through analysis of genome sequences [16]. However, the prevalence and primary mechanisms of PhO resistance still need to be clarified in this zoonotic pathogen.

Bacitracin, a polypeptide antibiotic, is used extensively in topical applications for prophylaxis and therapy in food animals. Recent studies characterized an acquired bacitracin resistance locus *bcrABDR* encoding high-level bacitracin resistance as the primary resistance mechanism in *E. faecium* [17] and *Enterococcus faecalis* [18]. However, Pires et al. [19] and Tsuda et al. [20] did not detect the related *bcrABDR* locus among bacitracin-resistant *Streptococcus pyogenes* and *Streptococcus mutans*, respectively. It is not yet known

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Table 1
Minimum inhibitory concentrations (MICs) of SFJ44 and identified resistance mechanisms known to confer resistance to antimicrobial agents

Classes	Antibiotics	MICs (mg/L)	Phenotype[a]	Resistance mechanisms
Penicillin	Penicillin	4	R	87% identity of PBP2X to P1/7
	Amoxicillin	2	R	87% identity of PBP2X to P1/7
Cephalosporin	Ceftiofur	1	Elevated MIC values	Compared with P1/7
Fluoroquinolone	Ciprofloxacin	8	R	Mutations in GyrA (Ser81 to Lys) and in ParC (Ser79 to Tyr)[b]
	Enrofloxacin	8	R	Mutations in GyrA (Ser81 to Lys) and in ParC (Ser79 to Tyr)[b]
Aminoglycoside	Gentamicin	16	Not HLGR	-
	Streptomycin	>256	HLSR	*aadE, sat4*
	Spectinomycin	256	R	*spw*
	Kanamycin	128	R	*aphA3*
Tetracycline	Tetracycline	128	R	*tet*AB(P)', *tet*(M)
Macrolide	Erythromycin	>256	R	*mef*(A), *msr*(D), *erm*(A)-like, *erm*(B)
	Tilmicosin	>256	R	*erm*(A)-like, *erm*(B)
	Tulathromycin	256	R	*erm*(A)-like, *erm*(B)
Lincosamide	Lincosamicin	>256	R	*erm*(A)-like, *erm*(B), *cfr*
	Clindamycin	>256	R	*erm*(A)-like, *erm*(B), *cfr*
Pleuromutilin	Valnemulin	32	R	*cfr*
Phenicol	Chloramphenicol	16	R	*optrA, cfr, catQ*
	Florfenicol	8	R	*optrA, cfr*
Oxazolidinone	Linezolid	4	R	*optrA, cfr*
Polypeptide	Bacitracin	>256	R	*brcABDR*

HLGR, high-level gentamycin resistance; HLSR, high-level streptomycin resistance.
[a] The MIC breakpoint accords to the guidelines for *Streptococcus suis* or viridans streptococci of the Clinical Laboratory Standards Institute (VET01-A4 or M100-S26) and the European Committee on Antimicrobial Susceptibility Testing.
[b] Amino acid positions correspond to those of *Streptococcus pneumoniae* R6.

if the genes are responsible for high-level bacitracin resistance in streptococci, including *S. suis*.

This study identified and characterized an extensively-drug-resistant (XDR) *S. suis* isolate which carried the PhO resistance genes *optrA* and *cfr*, and the bacitracin resistance locus *bcrABDR*. To the best of the authors' knowledge, this is the first report of the co-existence of *optrA* and *cfr*, and of the *bcrABDR* locus in streptococci.

2. Materials and methods

2.1. Bacterial isolation and PhO resistance mechanisms

In total, 107 *S. suis* isolates were collected from nasal swabs of healthy pigs or asymptomatic carriers for surveillance of PhO resistance in Jiangsu, China in 2016–2017. The project aims to survey the current status of oxazolidinone resistance in *S. suis* from healthy pigs. All isolates were identified by polymerase chain reaction (PCR) using primers targeting the *gdh* gene, as described previously [21]. Isolates with a minimum inhibitory concentration (MIC) value of florfenicol ≥8 mg/L and linezolid MIC value ≥4 mg/L were screened for the presence of PhO resistance genes *cfr, cfr*(B), *cfr*(C), *optrA* and *poxtA*.

2.2. Antimicrobial susceptibility testing

Antimicrobial susceptibility was determined using the broth microdilution method following Clinical Laboratory Standards Institute document VET01-A4 and European Committee on Antimicrobial Susceptibility Testing guideline (http://www.eucast.org). The antimicrobial agents used are summarized in Table 1.

2.3. Whole-genome sequencing and analysis

Genomic DNA was purified and subjected to sequencing by the Illumina HiSeq2500 platform, followed by PacBio SMRT technology at Novogene Bioinformatics Technology Co., Ltd. Acquired resistance genes and chromosomal mutations in genes known to be involved in antibiotic resistance were identified using BLASTn analysis against ResFinder database [22]. Antibiotic-resistance-associated genomic islands (ARGIs) were identified by comparison of SFJ44 with the available complete genomes of *S. suis* serotypes 1, 1/2, 2, 3, 4, 7, 9, 14, 16, 31 and Chz. The circular intermediate of IS-mediated resistance genes was determined by PCR.

2.4. Conjugation assays

Conjugation assays were examined by filter mating experiment, as described previously [23]. In mating experiments, donor and recipient strains were mixed at a ratio of 1:10 on a nitrocellulose membrane. Selection of transconjugants was performed on Todd-Hewitt agar containing appropriate antibiotics. *S. suis* P1/7RF was used as the recipient strain.

2.5. Zebrafish infection model

Zebrafish were challenged with *S. suis* strains as described previously [24]. Several groups of 15 zebrafish were incubated in plastic containers at 28°C for 72 h, and survival was scored from three parallel experiments. For determination of the median lethal doses (LD_{50}), a series of 10-fold serial dilutions [10^5 to 10^8 colony-forming units (cfu)] were injected, and the LD_{50} values at 72 h were calculated by the Reed and Muench method [25]. An *S. suis* serotype 2 hypervirulent strain SC070731 and phosphate buffered saline served as controls.

2.6. Nucleotide sequence accession number

The complete genome of *S. suis* SFJ44 has been deposited in GenBank (Accession No. CP031970).

3. Results and discussion

3.1. Increasing PhO resistance in S. suis

Between 2016 and 2017, 107 *S. suis* isolates were identified from eight pig farms in Jiangsu Province, China. Forty-one isolates (38.3%) were resistant to florfenicol and linezolid, of which all were positive for *optrA*. Among them, one isolate was also positive for *cfr*, and all isolates were negative for *cfr*(B), *cfr*(C) and *poxtA*. These results indicate the emergence and rapid increase of PhO resistance

(from ~0 to 38.3%), conferred by recent acquisition of *optrA*, probably between 2013 and 2016, compared with previous antimicrobial resistance data in Jiangsu, China [16,26–28].

3.2. Identification of the XDR S. suis isolate and its resistance mechanisms

A single isolate, SFJ44, was found to carry both *optrA* and *cfr*, as well as other resistance genes. It exhibited resistance to all 20 of the antimicrobial agents tested, except for ceftiofur, although elevated MIC values for ceftiofur were observed compared with *S. suis* P1/7 (Table 1). This isolate was categorized as XDR based on the definition for *S. suis* developed recently in veterinary medicine [29]. The complete genome of XDR isolate SFJ44 was 2 026 344 bp in length, with an average GC content of 39.34%. Sequence analysis showed that SFJ44 was non-serotypeable and belonged to a new sequence type ST-1087.

To further clarify the antibiotic resistance mechanisms, a BLAST search for acquired antibiotic resistance genes in the genome of *S. suis* SFJ44 was performed. In addition to *optrA* and *cfr*, other resistance genes were found, including: streptomycin resistance genes *aadE* and *sat4*; spectinomycin resistance gene *spw*-like; kanamycin resistance gene *aphA3*; macrolide-lincosamide-streptogramin B resistance genes *mef*(A), *msr*(D), *erm*(A)-like and *erm*(B); tetracycline resistance genes *tetAB*(P)' and *tet*(M); and chloramphenicol resistance gene *catQ* (Table 1). These findings are among the first to report that an ABC transporter gene locus *bcrABDR*, which conferred bacitracin resistance in *Enterococcus* spp. and *Clostridium perfringens* [18,30], also existed in *S. suis* and had high-level resistance to bacitracin with MIC >256 mg/L. As bacitracin is used extensively in topical applications with staphylococcus and streptococci infections, emergence of the *bcrABDR* locus in *S. suis* may become a therapeutic problem, as it has been suggested that this species may act as an antibiotic resistance reservoir contributing to the spread of resistance genes to major streptococcal pathogens [31,32].

Chromosomal mutations involved in resistance to β-lactams [33] and substitution of resistance to fluoroquinolones [34,35] in GyrA (Ser81 to Lys) and ParC (Ser79 to Tyr) were also observed [Table 1 and Fig. S1 (see online supplementary material)]. The resistance phenotype is in agreement with the antimicrobial resistance genotype, mediated by acquired resistance genes and chromosomal mutations.

3.3. Genetic environments of bcrABDR, optrA and cfr

To further characterize the genetic environment of the resistance genes, the ARGIs were mapped by comparing the genomes of SFJ44 with other representative *S. suis* strains of serotypes 1, 1/2, 2, 3, 4, 7, 9, 14, 16, 31 and Chz (Fig. S2, see online supplementary material). Comparative analysis revealed the presence of four ARGIs (designated ARGI1–ARGI4) and a type I-C CRISPR locus (Fig. S2, see online supplementary material). The size, insertion location, *att* site and resistance genes carried by ARGIs are summarized in Table S1 (see online supplementary material).

The 43 782-bp ARGI1 spanned the region from downstream of fructose-bisphosphate aldolase gene *fba* (SSU0312 of *S. suis* strain P1/7) to upstream of ribosome maturation factor gene *rimP* (SSU1646). It encoded 54 putative ORFs and carried four classes of antibiotic resistance genes, the *bcrABDR* locus, the *optrA*-*erm*(A)-like segment and the *apt*-*spw*-like cluster (Fig. S3, see online supplementary material).

The 3 287-bp *bcrABDR* locus on ARGI1 was almost identical (>99.9%) to the corresponding sequences of *E. faecalis* plasmid pEF123 (KX579977), *E. faecium* plasmid pXD5 (KJ645709), *Streptococcus pyogenes* NGAS322 genome (CP010449) and *Staphylococcus aureus* FDAARGOS_37 genome (JYAO02000004) [17,36]. Interestingly, the above *bcrABDR* locus was flanked by two copies of the IS*6* family insertion sequence IS*Enfa1* located in the same orientation, and was oriented in the opposite direction to IS*Enfa1*, presenting an IS*Enfa1*-*bcrABDR*-IS*Enfa1* pattern (Fig. 1A). However, upstream of the *bcrABDR* locus in *S. suis* SFJ44, the IS sequence was closer to IS*1216E* (99.4%) than IS*Enfa1* (81.4%), thus possessing an IS*1216E*-*bcrABDR*-IS*Enfa1* pattern (Fig. 1A). The *bcrABDR* locus also showed 95.3% sequence identity to *Clostridium perfringens* pJIR4150 (LN835295), where IS*Enfa1* was found neither upstream nor downstream of the *bcrABDR* locus in the latter's plasmid (Fig. 1A), which speculated derival from IS*Enfa1*-*bcrABDR*-IS*Enfa1* after the loss of IS*Enfa1*.

The *optrA*-carrying fragment on ARGI1 from the truncated putative transcriptional regulator gene Δ*araC* to S-adenosylmethionine-dependent methyltransferase gene *met* showed 99.9% (6252/6253) identity to *S. suis* YS57 (ALMZ01000078) and 99.7% (6272/6290) identity to *E. faecalis* LY4 (KT862785), while the *optrA* region exhibited 99.9% (3528/3531) identity to *E. faecalis* plasmid pE394 (KP399637) and 99.1% (2397/2417) identity to *S. aureus* plasmid pwo28-3 (KT601170) (Fig. 1B). The OptrA protein from *S. suis* SFJ44 showed two amino acid differences at positions 176 (Tyr→Asp) and 393 (Gly→Asp) from *E. faecalis* pE394. OptrA proteins with these substitutions have been proven to be functional in oxazolidinone resistance in a previous study [12].

ARGI4 was 57 542 bp in size and encoded 63 putative ORFs by integrated into the 3′-terminal of *tRNA-Leu* locus (Fig. S3, see online supplementary material). A 15 direct repeat sequence (5′-actataccggcggcc-3′) was found in both sites (*attL1* and *attR*) and an internal region (*attL2*) of ARGI4, resulting in a left fragment (10.6-kb) and a larger element of 46.9 kb. The latter carried the multiresistance gene *cfr* and putative Type I restriction-modification system locus *hsdRMS*. The *hsdRMS* locus exhibited 96.8% identity to the corresponding sequence of *Lactococcus lactis* plasmid pVF21 (JN172911).

The *cfr* and downstream ΔIS*Enfa5* on ARGI4 showed significant identity to corresponding sequences of *E. faecalis* plasmid pEF-01 [5] and *S. suis* plasmid pStrcfr [15] (2945 and 2943 of 2946, respectively), and *S. aureus* plasmid pSCFS3 [37] (1373/1376) (Fig. 1C). Immediately upstream of *cfr*-ΔIS*Enfa5*, an IS*6* family insertion sequence IS*1216E* and an integrase gene were detected. Just downstream of *cfr*-ΔIS*Enfa5*, there was another IS*6* family insertion sequence IS*Teha2*, which showed 75.5% (585/775) and 83.1% (188/226) identity with the upstream IS*1216E* at nucleotide and amino acid levels, respectively. The high homology between IS*1216E* and IS*Teha2* suggests that a different circular form generated from the IS*1216E*-*cfr*-ΔIS*Enfa5*-IS*Teha2* segment may exist. To determine this hypothesis, an inverse PCR assay was performed using previously designed primers [15], but no amplicon was observed.

3.4. Transferability of ARGIs

To test transferability of the ARGIs, conjugation assays using *S. suis* SF44 as the donor and *S. suis* P1/7RF as the recipient were performed, but no transconjugants were obtained after three attempts. Research is needed to determine how *S. suis* isolates acquire these resistance genes and whether the resistance genes/ARGIs can be transferred. Nevertheless, an inverse PCR and sequence analysis described previously [17,36] confirmed the formation of circular intermediates of *bcrABDR*-IS*Enfa1* in *S. suis* SFJ44 (data not shown). The present results, together with those from previous studies, indicate that IS*Enfa1*, possibly downstream, may be a critical element for intra- and interspecific transmission of *bcrABDR*-mediated bacitracin resistance in plasmids or chromosomes among Gram-positive bacteria [17,36]. Further, insertion of these resistance genes into the *S. suis* chromosome enable it to persist, which is

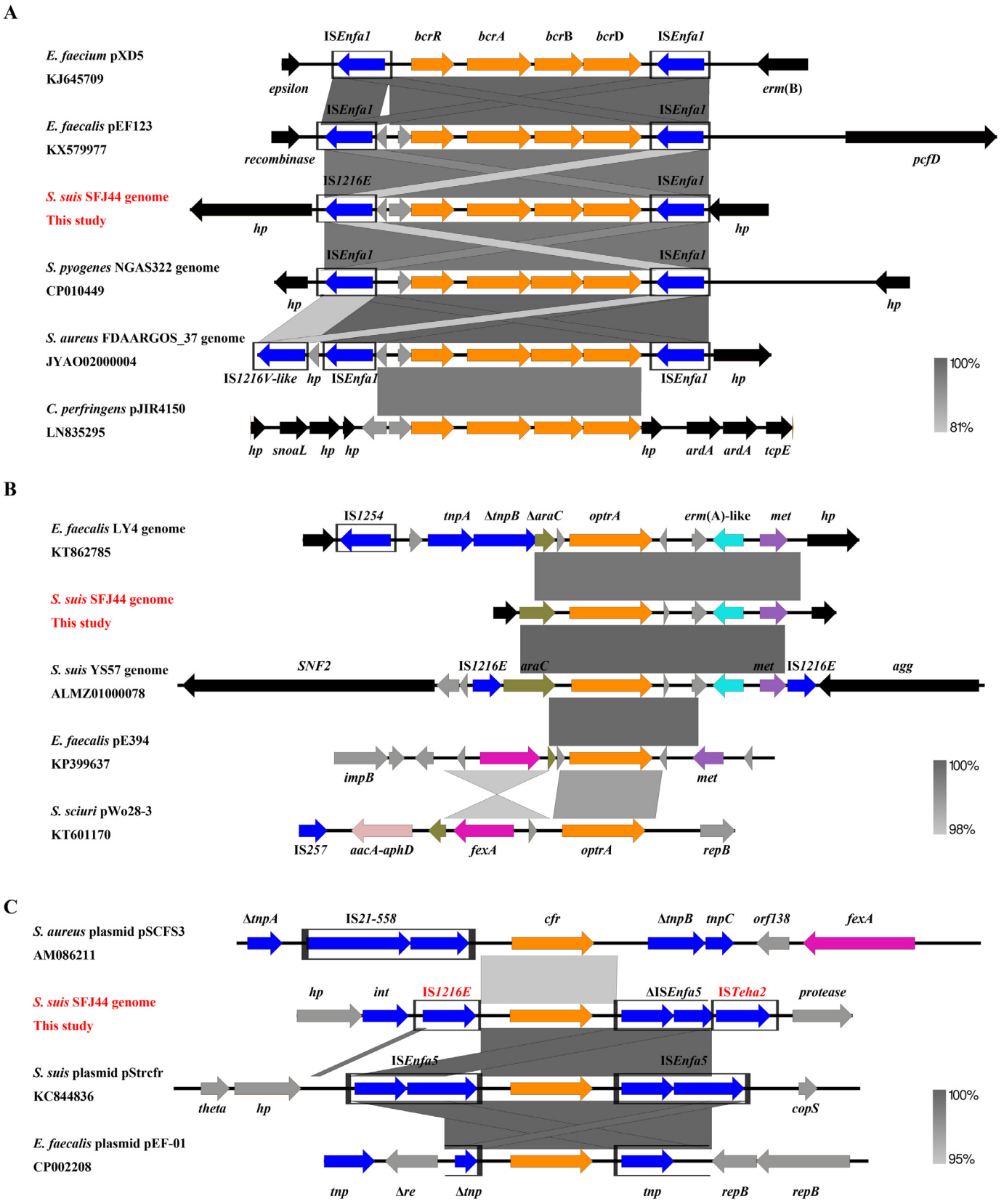

Fig. 1. Genetic contexts of the *bcrABDR* locus, *optrA* and *cfr* of *Streptococcus suis* SFJ44 with corresponding sequences reported in other Gram-positive bacteria. (A) Genetic environment of *S. suis* SFJ44 genomic *bcrABDR* locus in comparison with *Enterococcus faecium* plasmid pXD5, *Enterococcus faecalis* plasmid pEF123, *Streptococcus pyogenes* NGAS322 genomic, *Staphylococcus aureus* FDAARGOS_37 genomic and *Clostridium perfringens* plasmid pJIR4150 sequences. (B) Genetic environment of *S. suis* SFJ44 genomic *optrA* region in comparison with *E. faecalis* LY4 genomic, *S. suis* YS57 genomic, *E. faecalis* plasmid pE394, and *Staphylococcus sciuri* plasmid pWo26-2 sequences. (C) Genetic environment of *S. suis* SFJ44 genomic *cfr* region in comparison with *S. aureus* plasmid pSCFS3, *S. suis* plasmid pStrcfr and *E. faecalis* plasmid pEF-01 sequences.

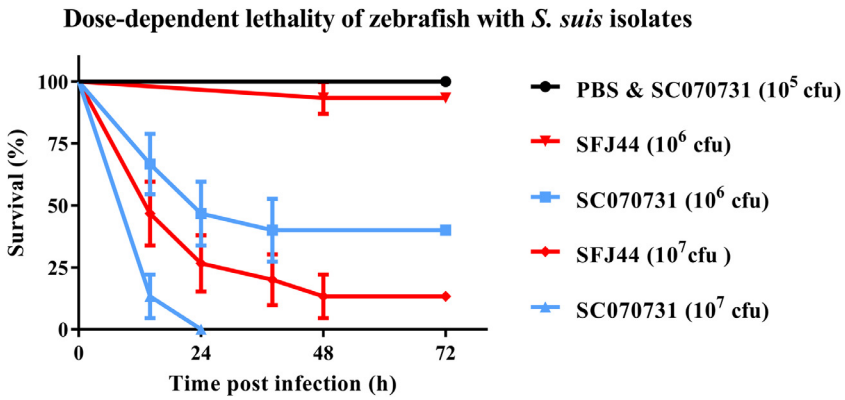

Fig. 2. Dose-dependent lethality of zebrafish model infected with *Streptococcus suis* isolate SFJ44 and a serotype 2 hypervirulent reference strain SC070731. Zebrafish were injected with serial dilutions of colony-forming units of *S. suis*. The survival rate was recorded over a 72-h period after infection. Mean values from triple experiments with 15 zebrafish in each group are shown. Error bars represent the standard error of the mean.

becoming more prevalent and have led to the emergence of multidrug-resistant (MDR) or even XDR isolates in this zoonotic pathogen.

3.5. Virulence of the XDR isolate

SFJ44 was non-serotypeable based on PCR and the capsular polysaccharide synthesis gene locus [38,39]. The most prevalent virulence markers (*epf*, *mrp* and *sly*) in serotype 2 were absent in SFJ44, although six virulence-related genes (*fbps*, *gdh*, *gapdh*, *ciaRH*, *manN* and *purD*) were detected (Table S2, see online supplementary material). In order to evaluate the virulence of the non-serotypeable strain SFJ44, a zebrafish in-vivo infection model was employed. Only 6.67% (1/15) mortality was observed after 72 h with a dose of 10^6 cfu, whereas 86.67% (13/15) of zebrafish died with 10^7 cfu (Fig. 2). Injection of *S. suis* serotype 2 hypervirulent strain SC070731 resulted in 60% and 100% mortality with 10^6 and 10^7 cfu, respectively. LD_{50} was 1.21×10^6 cfu/fish for SFJ44 and 0.83×10^6 cfu/fish for SC070731. These results indicate that the non-serotypeable strain SFJ44 had a virulence level comparable to *S. suis* serotype 2 hypervirulent strain SC070731, highlighting the need for surveillance of the pathogenicity of multi-drug-resistant *S. suis* isolates.

4. Conclusions

This study showed that *optrA* played an important role in the increasing incidence of PhO resistance among *S. suis* and described firstly the co-existence of *optrA* and *cfr* genes in the chromosome in streptococci. Moreover, this is the first report of the *bcrABDR* locus in *S. suis*. The co-occurance of *optrA*, *cfr*, *bcrABDR* locus and other resistance genes within genomic islands might lead to the co-selection of these resistance genes, contributing to its persistence and accelerating its dissemination. As *S. suis* may act as an antibiotic resistance reservoir contributing to the spread of resistance genes to major streptococcal pathogens [31,32], the potential dissemination of these resistance genes among Gram-positive bacteria, especially in common pathogens of human and animal origin, such as enterococci, staphylococci and streptococci, is of concern and routine surveillance should be strengthened.

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Competing interests

None declared.

Ethical approval

The zebrafish infection experiment was carried out in the Laboratory Animal Center of Nanjing Agricultural University according to animal welfare standards and approved by the Department of Science and Technology of Jiangsu Province (Permit number: SYXK (Su) 2017-0007).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2019.04.003.

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