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Colocation of the Multiresistance Gene *cfr* and the Fosfomycin Resistance Gene *fosD* on a Novel Plasmid in *Staphylococcus arlettae* from a Chicken Farm

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ABSTRACT The novel 63,558-bp plasmid pSA-01, which harbors nine antibiotic resistance genes, including *cfr*, *erm*(C), *tet*(L), *erm*(T), *aadD*, *fosD*, *fexB*, *aacA-aphD*, and *erm*(B), was characterized in *Staphylococcus arlettae* strain SA-01, isolated from a chicken farm in China. The colocation of *cfr* and *fosD* genes was detected for the first time in an *S. arlettae* plasmid. The detection of two IS*431*-mediated circular forms containing resistance genes in SA-01 suggested that IS*431* may facilitate dissemination of antibiotic resistance genes.

KEYWORDS *Staphylococcus arlettae*, *cfr*, *fosD*, IS*431*, circular form

Staphylococcus arlettae is a member of the coagulase-negative staphylococci (CoNS) that can serve as a reservoir for various resistance genes, including *cfr*, and may facilitate the dissemination of resistance genes between different staphylococcal species or even between staphylococci and other bacterial genera (1, 2). The multiresistance gene *cfr*, which mediates resistance to five antimicrobial classes, including phenicols, lincosamides, oxazolidinones, pleuromutilins, and streptogramin A (3), was first described in 2000 (4) and has been identified in a number of staphylococcal species (1, 2, 4–8). Plasmids seem to play an important role in the intra- and intergenus transfer of this gene (9). Thus far, many *cfr*-carrying plasmids have been described, which differed in structure, size, and presence of additional resistance genes (7). Most of them carry additional resistance genes coding for phenicol resistance (*fexA*, *fexB*) (5, 6, 10), macrolide-lincosamide-streptogramin B (MLS_B) resistance [*erm*(B), *erm*(C), *erm*(33)] (6, 11, 12) or gentamicin-kanamycin-tobramycin resistance (*aacA-aphD*) (6). Lately, fosfomycin has gained attention, as it has remained active against both Gram-positive and Gram-negative multidrug-resistant bacteria (13). To date, some fosfomycin resistance genes have been described in various bacteria (13–15). The *fosD* gene, which mediated fosfomycin resistance, was reported previously in *Staphylococcus aureus* (16) and *Staphylococcus rostri* (1). In this study, we reported a novel multiresistance plasmid cocarrying *cfr* and *fosD* in *S. arlettae*.

S. arlettae isolate SA-01, identified by the BD Phoenix-100 diagnostic systems (Sparks, MD), was collected from a fecal sample from a commercial chicken farm in China in July 2015. Antimicrobial susceptibility testing, performed according to the protocols of the Clinical and Laboratory Standards Institute (CLSI) (17–19), indicated that it exhibited high MIC values for florfenicol (>256 mg/liter) and fosfomycin (>128 mg/liter) and showed a linezolid MIC value of 16 mg/liter (Table 1). PCR analysis confirmed that it carried the florfenicol resistance genes *cfr*, *fexA*, and *fexB*, using previously described primers (5, 20) and

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TABLE 1 MICs for *S. arlettae* SA-01, *S. aureus* RN4220, and the *S. aureus* RN4220 transformants carrying plasmid pSA-01

Bacterial isolate	MICs (mg/liter) of[a]:							
	FFC	CHL	ERY	KAN	FOF	TET	CIP	LZD
SA-01	>256	>64	>128	128	128	128	>64	16
S. aureus RN4220	4	4	0.25	0.25	≤1	≤0.5	0.5	2
Transformant RN4220+pSA-01	>256	>64	>128	128	128	32	0.5	16

[a]FFC, florfenicol; CHL, chloramphenicol; ERY, erythromycin; KAN, kanamycin; FOF, fosfomycin; TET, tetracycline; CIP, ciprofloxacin; LZD, linezolid.

the fosfomycin resistance gene *fosD*, using primers F1 (5′-AACTCTAACTTGTGTCCGTCAG-3′) and F2 (5′-GTGGCTTATGGGTTGCGTTA-3′).

Conjugation by filter mating (21) and electrotransformation using purified plasmid DNA were performed with *S. arlettae* SA-01 as donor and *S. aureus* RN4220 (22) as recipient. Florfenicol (10 mg/liter) or fosfomycin (32 mg/liter) was used as a selection marker. Conjugation assays were failed, but electrotransformation of the plasmid DNA from SA-01 to RN4220 was successful. Compared with RN4220, the transformant (designated RN4220+pSA-01) exhibited drastically increased MICs for florfenicol (>256 mg/liter), erythromycin (>128 mg/liter), kanamycin (128 mg/liter), and fosfomycin (128 mg/liter) (Table 1). PCR results revealed that *cfr*, *fexB*, and *fosD* were detected in the transformant RN4220+pSA-01.

Whole-genome sequencing for transformant RN4220+pSA-01 was performed on the Illumina MiSeq (Majorbio, Shanghai, China) using a 400-bp paired-end TruSeq library with a 2 × 300 run. The paired-end reads were assembled *de novo* using SOAP v2.04 and GapCloser v1.12. The gaps between different contigs were closed by PCR and sequencing. Sequence analysis was conducted using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). A 63,558-bp plasmid (designated pSA-01) with an average GC content of 31.9% was obtained (Fig. 1). The *cfr*, *fexB*, and *fosD* genes were colocated in pSA-01, which also harbored *erm*(C), *erm*(T), *erm*(B), *tet*(L), *aadD*, and *aacA-aphD* (Fig. 1). Cooccurrence of these genes may lead to the persistence and coselection of *cfr* under selective pressure imposed by the use of aminoglycosides, MLS_B compounds, tetracycline, or fosfomycin. Although *cfr* has been reported to coexist with *erm*(B) (6, 11), *erm*(C) (6), *fexB* (12), or *aacA-aphD* (6) and so on, to our knowledge, this is the first report for colocation of *cfr* and *fosD* in a plasmid from *S. arlettae*.

Based on its genetic content, pSA-01 was divided into two regions, A and B (Fig. 1). Region A was 22,933 bp in size and consisted of the backbone of pSA-01. Three replication genes, including *repA* and its flanked genes, showed over 97% identity to corresponding regions of plasmid pStO2014-01 from *Staphylococcus condimenti* (GenBank accession no. CP018777) and pC2014-3 from *Staphylococcus equorum* (accession no. CP013717), respectively. These genes are essential for plasmid replication. The products of *merR1* and *merA* showed 94% and 90% identity to proteins MerR1 and MerA of the mer operon in *Bacillus* sp. YR31 (accession no. LC015493), respectively. The genes *arsB* and *arsC* showed 85% and 86% identity to the gene coding for the arsenic transporter of *Sporosarcina psychrophila* (accession no. CP014616) and the gene coding for arsenate reductase of *Staphylococcus equorum* (accession no. CP013714), respectively. These genes are associated with heavy-metal (mercury and arsenic) resistance (23, 24). Additionally, the remaining 11 genes located in the backbone, including hypothetical protein genes, appear to have been derived from various sources, as their deduced amino acid identities ranged from 70% to 99% to corresponding proteins of *Staphylococcus* spp., *Bacillus* spp., and *Sporosarcina* spp.

The 40,625-bp region B (nucleotides [nt] 16,317 to 56,941) harbored nine resistance genes, which were carried by segments originating from various sources (Fig. 2). The *cfr–erm*(C)-carrying segment, which showed 99.5% identity to plasmid pSS-03 (accession no. JQ219851) (6), was found to be inserted into a 4,413-bp fragment showing 99.8% identity to an *Enterococcus faecium* plasmid (accession no. CP011830) (25). This

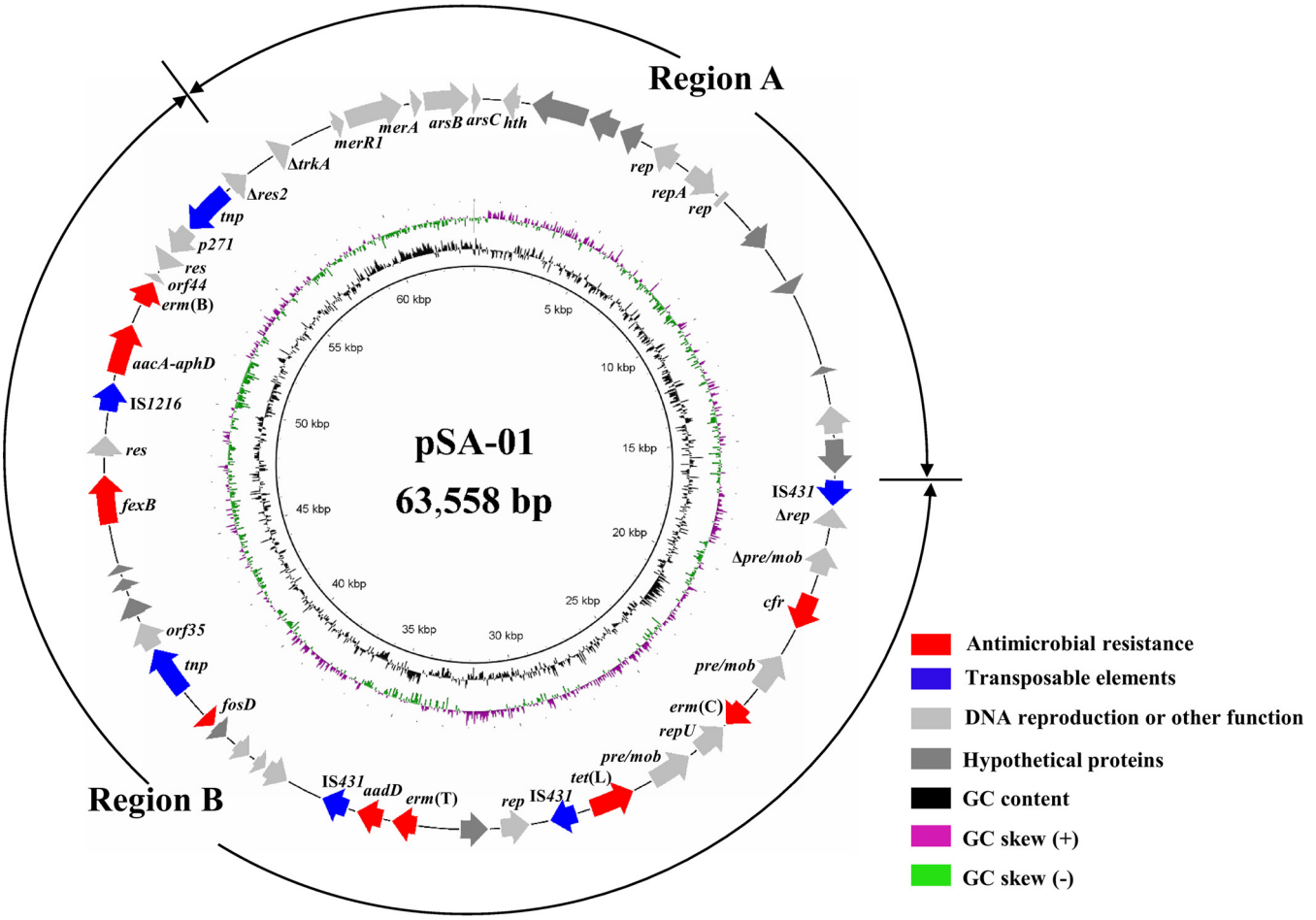

FIG 1 Genetic map of pSA-01. Positions and directions of predicted open reading frames are indicated by colored arrows according to their predicted functions. Truncated coding sequences (CDS) are indicated with a Greek delta symbol.

segment, spanning from Δ*rep* to *tet*(L), was flanked by two IS*431* copies (Fig. 2). Moreover, a 7,240-bp segment comprising *erm*(T) and *aadD* was also bracketed by IS*431* and showed 99% identity to the chromosomal integrated plasmid pUR3912 (accession no. HF677199) (26, 27) with 80% query coverage. IS*431* seemed to mediate the integration of this segment, since pUR3912 could integrate into the chromosomal DNA via IS*431* (26). An 8,647-bp fragment containing *fosD* and *fexB* seemed to insert into a 7,400-bp *aacA–aphD–erm*(B)-carrying fragment, which showed 99.8% identity to the corresponding region of pNTUH_3874 (accession no. LC102479) (28). Within the 8,647-bp fragment, the *fosD*-carrying fragment (1,002 bp) exhibited over 99.9% identity

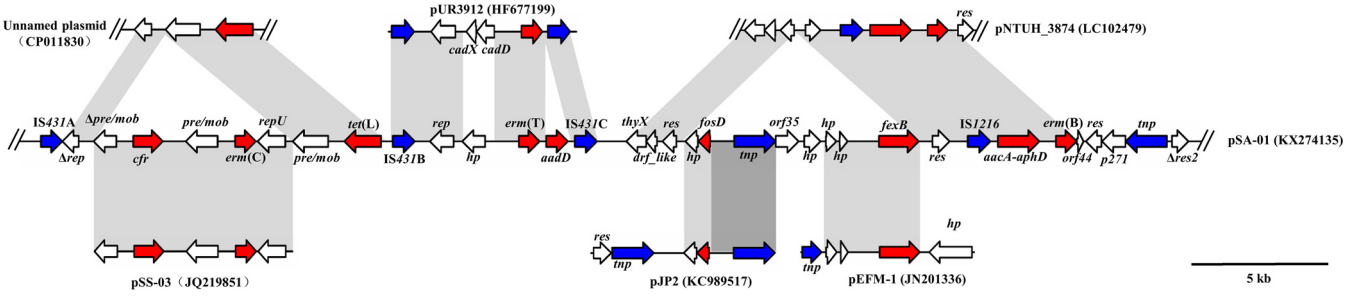

FIG 2 Schematic presentation of region B in plasmid pSA-01 in comparison with other plasmids. Regions of >99% nucleotide sequence identity are marked in light gray, while dark gray represents region of 91.2% nucleotide sequence identity. Arrows indicate the positions and orientations of the genes.

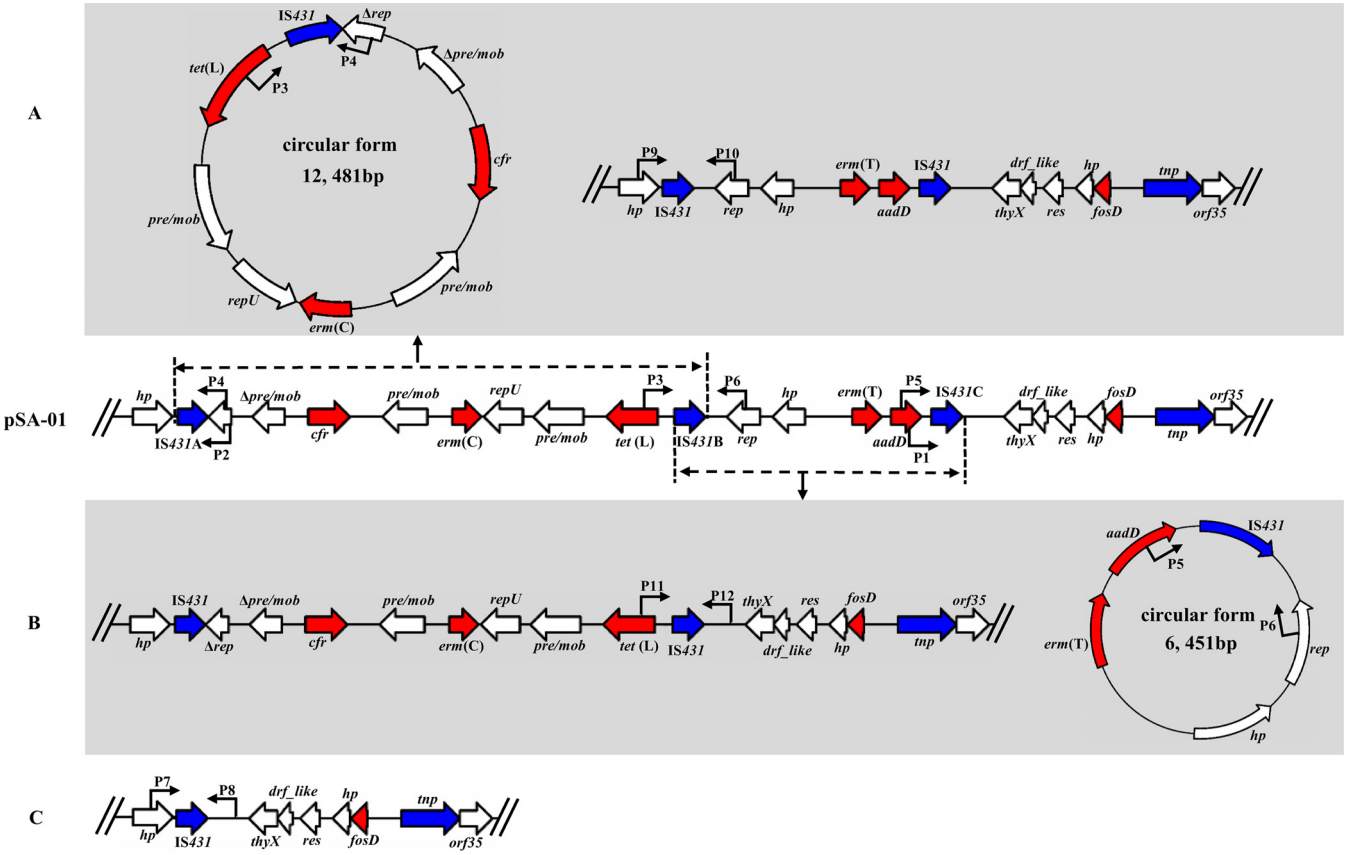

FIG 3 Formation of circular forms mediated by IS*431*. (A) Circular form derived from the region spanning from IS*431*A to IS*431*B and the structure that missed the corresponding region of the circular form. (B) Circular form derived from the region spanning from IS*431*B to IS*431*C and the structure that missed the corresponding region of the circular form. (C) Structure that missed the region spanning from Δ*rep* to IS*431*C. The locations and orientations of primers (P1 to P12) are indicated by arrows. Primers P1 to P6 were used for inverse PCR to detect circular forms (primers P3 and P4 for the 12,481-bp circular form and P5 and P6 for the 6,451-bp circular form; inverse PCR with primers P1 and P2 produced no product); primers P7 to P12 were used to detect structures that missed the corresponding region of the circular form (P7 and P8 for structure that missed the region spanning from Δ*rep* to IS*431*C; P9 and P10 for structure that missed the corresponding region of the 12,481-bp circular form; and P11 and P12 for structure that missed the corresponding region of the 6,451-bp circular form).

to the corresponding region of pJP2 (accession no. KC989517) (1), but downstream of *fosD* a 2,320-bp segment containing a transposase gene showed just 91% identity to pJP2. The 3,653-bp *fexB*-containing segment showed 99.9% identity to plasmid pEFM-1 from *Enterococcus faecium* (accession no. JN201336) (20). The common DNA segments in these different plasmids suggested recombination between plasmids of different pathogens; pSA-01 was a complex and hybrid plasmid.

In pSA-01, the presence of multiple copies of insertion sequence (IS) (three IS*431*and one IS*1216*) were identified, which might facilitate intra- or interplasmid recombination. Three IS*431* copies (named IS*431*A, IS*431*B, and IS*431*C based on their positions) were in the same orientation. Since direct repeats of IS may mediate dissemination of genes via formation of the circular form (1, 10, 29), inverse PCR assays (see Table S1 in the supplemental material for primers) were performed to detect whether IS*431* mediated the formation of the circular form. Two circular forms of 12,481 bp and 6,451 bp were observed in SA-01 (Fig. 3). Both of the circular forms contained an intact IS*431* and the region between IS*431*A and IS*431*B (Fig. 3A) or region between IS*431*B and IS*431*C (Fig. 3B). To further confirm the formation of circular forms, PCR assays (primers shown in Table S1) were performed to detect the structures that missed the corresponding region of the circular form. The results of these PCR assays matched with inverse PCR. Interestingly, although no circular form was observed between IS*431*A and IS*431*C, the structure that missed regions spanning from Δ*rep* to IS*431*C was detected (Fig. 3C). This observation indicated that the two circular forms may form simultaneously. These

findings might suggest that the association of resistance genes with IS*431* facilitated their translocation. Besides, IS*1216* might also have been involved in the recombination of pSA-01, since it has been reported to play an important role in the dissemination of antimicrobial resistance determinants (30) and in plasmid recombination (31).

In conclusion, pSA-01 was a complex, hybrid multiresistance plasmid. As far as we know, the *fosD* gene was described in an *S. arlettae* plasmid for the first time. The coexistence of *cfr* with other resistance genes, especially *fosD*, will limit antimicrobial treatment options and may lead to coselection of these genes even in the absence of direct selective pressure. The structures bracketed by IS*431* were unstable and could be looped out by IS-mediated recombination. The presence of IS elements might facilitate intra- or interplasmid recombination and dissemination of resistance genes. Given that the presence of the *cfr*–*fosD*-carrying multiresistance plasmid may seriously compromise the effectiveness of clinical therapy and threaten public health, its occurrence and dissemination need further surveillance.

Accession number(s). The complete nucleotide sequence of the 63,558-bp plasmid pSA-01 characterized in this study was submitted to the GenBank database and assigned accession number KX274135.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.01388-17.

TABLE S1, PDF file, 0.4 MB.

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