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A Novel FexA Variant from a Canine *Staphylococcus pseudintermedius* Isolate That Does Not Confer Florfenicol Resistance

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Transposon Tn*558* integrated in the chromosomal *radC* gene was detected for the first time in *Staphylococus pseudintermedius*. It carried a novel *fexA* variant (*fexAv*) that confers only chloramphenicol resistance. The exporter FexAv exhibited two amino acid substitutions, Gly33Ala and Ala37Val, both of which seem to be important for substrate recognition. Site-directed mutagenesis that reverted the mutated base pairs to those present in the original *fexA* gene restored the chloramphenicol-plus-florfenicol resistance phenotype.

In staphylococci, phenicol resistance is mediated either by the chloramphenicol acetyltransferase-encoding *cat* genes, which confer resistance to nonfluorinated phenicols (e.g., chloramphenicol), or either of the two genes *fexA* (coding for a phenicol-specific efflux pump) and *cfr* (coding for a rRNA methyltransferase), both of which mediate combined resistance to fluorinated phenicols (e.g., florfenicol) and nonfluorinated phenicols (1). Since the first description of the *fexA* gene in a bovine *Staphylococcus lentus* isolate (2), this gene has been detected—either as part of the small nonconjugative transposon Tn*558* or in combination with the *cfr* gene in transposition-deficient Tn*558* variants—in *S. aureus* and several coagulase-negative staphylococcal species from healthy and diseased cattle, swine, horses, or humans (3–9). The *fexA* gene has also been detected in a *Bacillus* isolate from swine feces and in environmental pollutants from swine feedlots in China (10, 11).

In a previous study that focused on the occurrence of methicillin-resistant coagulase-positive staphylococci in dogs in La Rioja, Spain, one methicillin-susceptible *S. pseudintermedius* isolate, named C2719, was identified (12). This isolate was recovered from a healthy dog admitted to a veterinary clinic for a routine checkup. No additional information on the living conditions of the dog and/or the possible contact with rural areas or livestock was recorded. Susceptibility testing by agar disk diffusion and/or broth microdilution (13) showed that isolate C2719 was resistant to penicillin (due to the *blaZ* gene) and to chloramphenicol (MIC 64 μg/ml) but exhibited a low MIC of 2 μg/ml for florfenicol. None of the three *cat* genes known to occur in staphylococci—cat_{pC194}, cat_{pC221}, and cat_{pC223} (1)—was detected by PCR (14). The objective of this study was to identify the gene responsible for chloramphenicol resistance in this strain and to characterize its genetic environment.

PCR analysis for the chloramphenicol/florfenicol resistance genes *fexA* and *cfr* (5) showed that, despite its low florfenicol MIC, strain C2719 harbored the *fexA* gene. PCR mapping and sequencing revealed that this *fexA* variant, named *fexAv*, exhibited 99.7% nucleotide sequence identity (99.2% identity and 99.6% similarity at the protein level) to the prototype *fexA* gene of *S. lentus* (GenBank accession no. AJ549214) (2). Moreover, the *fexAv* gene was found to be part of the Tn*558* transposon (3, 15) (Fig. 1). Tn*558* is a member of the Tn*554* family (15). Transposons of this family exhibit several features that distinguish them from most other transposable elements: (i) their ends are asymmetric, lacking either inverted or direct terminal repeats, (ii) they do not generate a duplication of the target sequence upon transposition, and (iii) they are extremely site specific, almost always inserting into the staphylococcal chromosome at the same location. This unique target site *att*, is located within the *radC* gene, which codes for a DNA repair protein (16–22). In addition to Tn*558*, first described in *S. lentus* (15), this group also includes at least another four members: Tn*554* harboring the *erm*(A) gene for resistance to macrolides, lincosamides, and streptogramin B and the *spc* gene for spectinomycin resistance (19, 20); Tn*5406* carrying the *vga*(A) gene for resistance to lincosamides, pleuromutilins, and streptogramin A (16); Tn*559* harboring the *dfrK* gene for trimethoprim resistance (17); and Tn*6133*, which is composed of a Tn*554*-like element into which a segment with the novel gene *vga*(E) for resistance to lincosamides, pleuromutilins, and streptogramin A has been inserted (22). All these transposons have been found in *S. aureus*, with Tn*559* and Tn*6133* first detected in livestock-associated *S. aureus* of the lineage ST398.

The chromosomal/plasmid location of Tn*558*, as well as its specific integration site, was determined by plasmid preparation, specific PCRs, and sequencing (3, 23). As no plasmids were detected in *S. pseudintermedius* C2719, a chromosomal location of the *fexAv*-carrying transposon appeared to be likely. Primers radC_SP-fw and radC_SP-rv (Table 1) were designed to determine whether Tn*558* was integrated within the chromosomal *radC* gene. The target recognition sequence, *att558*, was identified on the basis of the nucleotide similarity of (i) the 6-bp "core" sequence (*att*, 5′-GATGTA-3′) of the *radC* gene of the original *S. lentus* isolate carrying Tn*558* (accession no. AJ715531) (15) and that detected on the *radC* gene of *S. pseudintermedius* HKU10-03 and (ii) the sequences flanking this *att* site, which are also essential for transposition (19, 20).

An amplicon of 7,082 bp that comprised the complete Tn*558*

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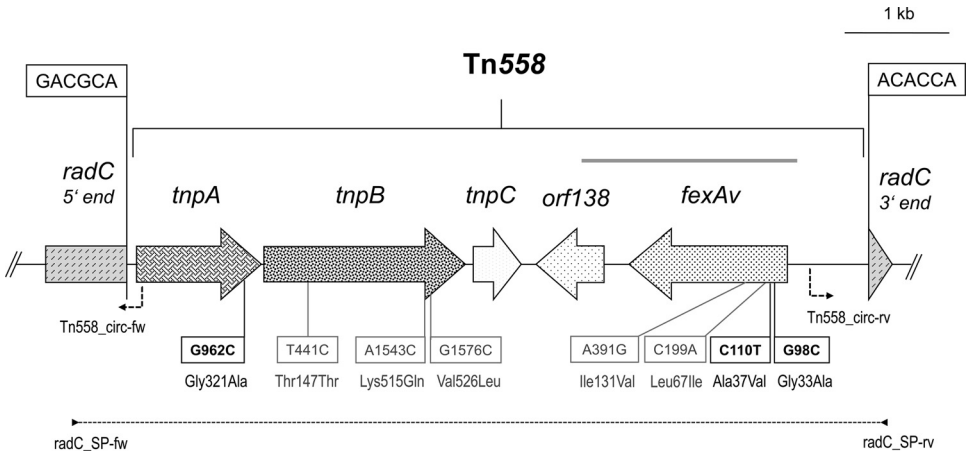

FIG 1 Schematic presentation of the Tn*558* structure carrying the *fexA*$_v$ gene detected in this study as well as its integration region (within the *radC* gene) in the chromosomal DNA of *S. pseudintermedius* C2719 (EMBL accession no. HF679552). The arrows indicate the extents and directions of transcription of chloramphenicol resistance (*fexAv*) and transposase (*tnpA*, *tnpB*, *tnpC*) genes as well as reading frame *orf138*. The 6-bp core nucleotide sequences at the transposon junctions are shown within boxes. The positions of primers used to amplify the region between the *radC* gene extremes are indicated as arrowheads, with a dashed line showing the extension length. The primers employed to detect circular intermediates, as well as the direction of amplification, are also shown. The gray bar over the *fexAv* gene indicates the region that has been used for cloning and mutagenesis experiments. A size scale in kilobases (kb) is displayed in the upper right-hand corner of the figure.

(6,645 bp) and part of the *radC* gene (437 bp) was obtained (Fig. 1). Complete analysis of the sequence of this transposon revealed 99.7% nucleotide identity to that of *S. lentus* (GenBank accession no. AJ715531). Sequence analysis identified three semiconserved amino acid substitutions in two reading frames: (i) Gly321Ala in the transposase protein TnpA (nucleotide G962C in the transposase gene *tnpA*) and (ii) Gly33Ala and Ala37Val in the FexAv protein (nucleotides G98C and C110T, respectively, within the *fexAv* gene) (Fig. 1). Four additional conserved amino acid substitutions were observed in Tn*558*: (i) Lys515Gln and Val526Leu in the TnpB transposase (nucleotides A1543C and G1576C, respectively) and (ii) Leu67Ile and Ile131Val within the FexAv protein (nucleotides C199A and A391G, respectively). No circular intermediates of Tn*558*, which are indicative for the mobility of the transposon, were detected by specific PCR (Fig. 1) (3) either when the bacteria were grown under normal growth conditions (13) or after growth under stress conditions (overnight cultures were exposed to ultraviolet light for 10 min or to a heat shock of 60°C for 2 h; growth of bacteria under conditions of anaerobiosis or at 45°C). Whether the observed amino acid changes in TnpA and/or TnpB or other factors account for the lack of mobility of Tn*558* under the tested conditions remains to be determined.

To confirm that the *fexAv* gene is in fact responsible for chloramphenicol but not for florfenicol resistance, a PCR assay using primers entire_fexA-1 and entire_fexA-2 (Table 1) that amplified the complete *fexAv* gene (1,428 bp), including 201 bp and 452 bp of its upstream and downstream region, respectively, was conducted. This 2,081-bp amplicon was first cloned into the pCR 2.1-TOPO vector and transformed into the recipient *Escherichia coli* TOP10 strain using a TOPO TA cloning kit (Invitrogen, Groningen, The Netherlands). Subsequent transformation into *E. coli* HB101 was conducted to test the functionality of the *fexAv* gene in

TABLE 1 Primers used to detect the chromosomal integration site of the *fexA*-carrying Tn*558* of *S. pseudintermedius* isolate C2719, those to amplify the complete *fexA* gene, and those to perform the site-directed-mutagenesis

Primer name	Primer sequence (5′→3′)[a]	Amplicon size (bp)	PCR conditions	Polymerase
radC_SP-fw	GTTTGTCGGAATAGGGCGTA	437[b]/7,082	1 min at 94°C; 30 cycles of 10 s at 98°C, 15 min at 68°C and 10 min at 72°C	La *Taq* (TaKaRa)
radC_SP-rv	ACGATTCTTCCCCAATCACA			
entire_fexA-1	GATCCGTAAGCCCATCCATA	2,081	3 min at 94°C; 30 cycles of 30 s at 94°C, 45 s at 55°C, and 2 min at 72°C; 5 min at 72°C	BioTaq (Bioline)
entire_fexA-2	AGGCACCGGTTGTTAAACTG			
fexA_33-inv1	ATCTGTACTTGTAG**G**TGCAATTACGGTTG	6,012[c]	3 min at 98°C; 20 cycles of 1 min at 98°C, 1 min at 55°C, and 2.5 min at 72°C; 10 min at 72°C	Phusion (Finnzymes)
fexA_33-inv2	CAACCGTAATTGCA**C**CTACAAGTACAGAT			
fexA_37-inv1	TAGCTGCAATTACGG**C**TGATTTAGTCAATCC			
fexA_37-inv2	GGGATTGACTAAATCA**G**CCGTAATTGCAGCTA			
fexA_33 + 37-inv1	CTATCTGTACTTGTAG**G**TGCAATTACGG**C**TGA TTTAGTCAATCCC			
fexA_33 + 37-inv2	GGGATTGACTAAATCA**G**CCGTAATTGCA**C**CTA CAAGTACAGATAG			

[a] Nucleotides in bold type and underlined are those modified by the site-directed-mutagenesis PCR.
[b] Amplicon size obtained when an intact copy of the *radC* gene was present.
[c] Size of the complete recombinant and mutated plasmid, composed of the PCR 2.1-TOPO vector (3,931 bp) and the insertion obtained with the entire_fexA-1 and entire_fexA-2 primers (2,081 bp).

a phenicol-susceptible *E. coli* host (chloramphenicol MIC, 2 μg/ml; florfenicol MIC, 4 μg/ml). *E. coli* HB101 carrying the recombinant vector exhibited a 16-fold increase in the chloramphenicol MIC (32 μg/ml), while the florfenicol MIC value remained unchanged.

In a study on the FloR chloramphenicol/florfenicol efflux protein, Braibant et al. (24) determined by site-directed mutagenesis (SDM) that the Asp23 residue seems to participate directly in the affinity pocket involved in phenicol-derivative recognition. When Asp23 was mutated to Glu23, the corresponding FloR protein still conferred chloramphenicol resistance but lost its ability to export florfenicol. A model (http://www.ch.embnet.org/software/TMPRED_form.html) of the transmembrane segments (TMS) of the 12-TMS FloR protein and the 14-TMS FexA protein predicted that the Asp23 residue in FloR and the Gly33/Ala37 residues in the prototype FexA protein are all located in transmembrane segment 1 and hence may have similar functions. Based on these data, SDM was conducted on the *fexAv* nucleotide substitutions G98C and C110T separately and on G98C and C110T in combination to revert them to those of the *fexA* prototype from *S. lentus*. For this, inverse PCR assays with specific "mutagenesis primers" were performed (Table 1). A plasmid preparation (Qiagen Plasmid Midikit; Qiagen, Hilden, Germany) of the aforementioned recombinant pCR 2.1-TOPO vector served as the template for SDM. After SDM, digestion of the obtained SDM products with the DpnI restriction enzyme (Fermentas, St. Leon-Rot, Germany) was performed to eliminate the original methylated template plasmid DNA. An aliquot from the SDM approach was then transferred by electrotransformation into *E. coli* HB101. Correctly mutated recombinant vectors (TOPO/$fexA_v^{C98G}$, TOPO/$fexA_v^{T110C}$, and TOPO/$fexA_v^{C98G+T110C}$) were confirmed by PCR of the complete *fexA* gene and sequence analysis of the amplicon (Table 1). Macrodilution assays for chloramphenicol and florfenicol (13) showed a florfenicol MIC of 16 μg/ml for the *E. coli* HB101 transformants carrying TOPO/$fexA_v^{C98G+T110C}$ that was 4-fold higher than that seen with the recipient strain alone (MIC 4 μg/ml) and clones carrying each of the reverted positions separately, while the chloramphenicol MIC remained unchanged in all mutants (MIC, 32 μg/ml). This increase in the florfenicol MIC was similar to that described for *E. coli* JM109 carrying the cloned *fexA* gene from *S. lentus* (2) and confirms that reversion of the naturally mutated positions restores the florfenicol resistance phenotype. It should be noted that the observed MIC changes might differ, at least slightly, in different bacterial hosts, such as in Gram-positive bacteria.

Nucleotide sequence accession number. The 7,698-bp nucleotide sequence enclosing the complete Tn*558* and the *radC* gene of *S. pseudintermedius* strain C2719 has been deposited on the EMBL database under accession number HF679552.

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