

# Identification of a Novel Trimethoprim Resistance Gene, *dfrK*, in a Methicillin-Resistant *Staphylococcus aureus* ST398 Strain and Its Physical Linkage to the Tetracycline Resistance Gene *tet(L)*<sup>∇</sup>

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**A novel trimethoprim resistance gene, designated *dfrK*, was detected in close proximity to the tetracycline resistance gene *tet(L)* on the ca. 40-kb plasmid pKKS2187 in a porcine methicillin (meticillin)-resistant *Staphylococcus aureus* isolate of sequence type 398. The *dfrK* gene encodes a 163-amino-acid dihydrofolate reductase that differs from all so-far-known dihydrofolate reductases.**

Methicillin (meticillin)-resistant *Staphylococcus aureus* (MRSA) represent a major problem in human medicine by causing both healthcare-associated and community-associated infections (5). A large number of MRSA clones has been identified (4, 11), one of which, consisting of strains of the sequence type 398 (ST398), has gained particular attention during recent years because of its association with pigs and its ability to colonize pig farmers and other people in close contact with pigs (18–21, 23). Although MRSA ST398 strains rarely cause infections in pigs, few reports described their involvement in specific disease conditions of pigs (15, 20). Recently, five multiresistant porcine MRSA ST398 strains from infections of the skin or the urinary/genital tract, including metritis-mastitis-agalactia syndrome, have been identified in the BfT-GermVet study (15, 16). PCR screening for antimicrobial resistance genes identified the  $\beta$ -lactam resistance gene *mecA* as well as the tetracycline resistance genes *tet(K)*, *tet(L)*, and/or *tet(M)* in various combinations in these isolates (16). In addition, three strains were resistant to gentamicin and kanamycin via the gene *aacA-aphD* [*aac(6')*-*Ie-aph(2')*-*Ia*], and one isolate was resistant to macrolides and lincosamides via the gene *erm(A)* (16). However, none of the known staphylococcal trimethoprim resistance genes, *dfrS1*, *dfrD*, and *dfrG*, could be detected by PCR in the three trimethoprim-resistant isolates.

One of these MRSA ST398 isolates, namely isolate 2187, was chosen for the identification of the presumably new trimethoprim resistance gene. This strain was resistant only to penicillins (penicillin G, ampicillin, and oxacillin [MICs of 8  $\mu$ g/ml]), tetracycline (MIC of 64  $\mu$ g/ml), and trimethoprim (MIC > 256  $\mu$ g/ml). Plasmid profiling and protoplast transformation assays were conducted as previously described (8). *Staphylococcus aureus* RN4220 served as the recipient strain, and transformants were selected on regeneration medium containing 10  $\mu$ g/ml trimethoprim. An analysis of the transformants revealed that a ca. 40-kb plasmid, designated pKKS2187, was associated with trimethoprim re-

sistance. The susceptibility testing and PCR analysis of the transformants carrying this plasmid confirmed that plasmid pKKS2187 also mediated tetracycline resistance via a *tet(L)* gene. To identify the pKKS2187-associated trimethoprim resistance gene, plasmid pKKS2187 was digested with BglII, and the resulting fragments were cloned into the BamHI site of pBluescript II SK+. *Escherichia coli* JM109 was transformed with these recombinant plasmids, and clones were selected by blue-white screening with subsequent cultivation on Luria-Bertani agar (Oxoid, Wesel, Germany) supplemented with 10  $\mu$ g/ml trimethoprim. Clones growing on these selective plates also exhibited tetracycline resistance and carried a common ca. 7-kb BglII fragment.

The sequence analysis of this 7,045-bp BglII fragment showed that its terminal parts consisted of IS257 sequences located in the same orientation (Fig. 1). Between these IS257 elements, a structure was detected that closely resembled the small *tet(L)*-carrying plasmid pBC16 with an additional segment that contained a novel trimethoprim resistance gene (Fig. 1). The *repU* gene was interrupted by the integration of the IS257 elements, and a typical 8-bp direct repeat, 5'-TGCTGA AA-3', was detected at the integration sites. The functional deletion of the *repU* gene ensured that the replication properties of the entire plasmid were specified solely by the original large plasmid without interference from the integrated small plasmid. This finding closely resembled the situation of small *tet(K)*-carrying plasmids being integrated via IS257 into larger staphylococcal plasmids (22).

Downstream of the 3' end of the *repU* gene in pKKS2187, a reading frame for a 458-amino-acid (aa) Tet(L) protein was found. This reading frame was preceded by a translational attenuator for inducible *tet(L)* gene expression that consisted of a small reading frame for a 20-aa peptide and two pairs of inverted repeated sequences. The *tet(L)* gene in pKKS2187 was indistinguishable from that of the tetracycline resistance plasmids pBC16 from *Bacillus cereus* (13), pTB19 from *Bacillus stearothermophilus* (12), and pLS1 from *Streptococcus agalactiae* (9). Another 282 bp downstream of the *tet(L)* gene, a reading frame for a 163-aa dihydrofolate reductase, designated DfrK, was detected. This gene mediated trimethoprim resistance, as confirmed by an at least 2,048-fold increase in the trimethoprim MIC to  $\geq 512$   $\mu$ g/ml for the recombinant *E. coli*

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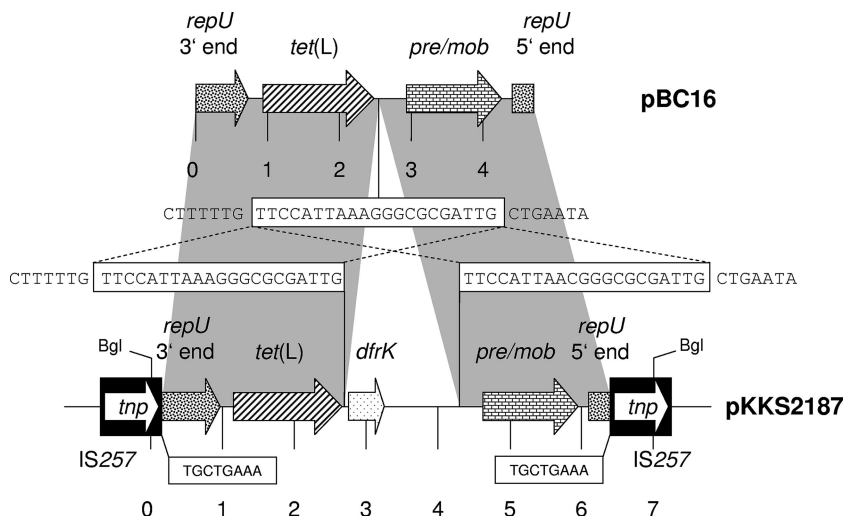


FIG. 1. Comparison of the *tet(L)-dfrK* segment of plasmid pKKS2187 (accession no. FM207105) with plasmid pBC16 from *B. cereus* (accession no. AAA84922). The arrows indicate the extents and directions of the transcription of the genes *repU* (plasmid replication), *tet(L)* (tetracycline resistance), *dfrK* (trimethoprim resistance), and *pre/mob* (plasmid recombination/mobilization). It should be noted that the *repU* gene of pBC16 is intact and has been displayed only in a disrupted form for better comparison to the pKKS2187 sequence. The IS257 elements are shown as black boxes, with the white arrow indicating the transposase gene *tnp*. The 8-bp direct repeats at the IS257 integration sites are shown in boxes. The regions of >99% homology between pBC16 and pKKS2187 are marked by gray shading. The sequences at the junctions of pBC16-homologous and -nonhomologous parts in pKKS2187 are shown for comparison to the corresponding pBC16 sequence between the two maps. The BglIII cleavage site in the IS257 element is indicated as Bgl. A size scale in kilobase pairs is given below each map.

JM109 clone carrying the 7-kb BglIII fragment compared to that of the original *E. coli* JM109 strain. An analysis of the *dfrK* nucleotide sequence revealed that this gene showed 86.2% identity to the gene *dfrG* from *Staphylococcus aureus* (17) and 81.2% identity to the *dfrD* genes found in *Staphylococcus haemolyticus* (3) and *Listeria monocytogenes* (1). On the amino acid level, identities of 87.9 and 77.2% were noted between DfrK and DfrG and between DfrK and DfrD, respectively. Distinctly lower levels of 49.3% nucleotide sequence identity between *dfrK* and the Tn4003-associated *dfrS1* gene (14) and 38.7% amino acid identity between DfrK and DfrS1 were seen. The phylogenetic tree shown in Fig. 2 confirmed that DfrD, DfrG, and DfrK form a separate branch that is only distantly related to DfrS1 and DfrC. DfrC is a trimethoprim-susceptible dihydrofolate reductase that is considered a potential precursor of the trimethoprim-resistant DfrS1 protein (2). The DfrB

proteins identified in MRSA strains (6, 7) also represent trimethoprim-susceptible dihydrofolate reductases.

Further downstream of *dfrK*, a reading frame for a 420-aa Pre/Mob protein indistinguishable from those of plasmids pBC16 (accession no. AAA84922) and pUB110 (10) was detected (Fig. 1). The entire integrated segment between the IS257 elements closely resembled the 4,630-bp plasmid pBC16. Two segments of 2,488 and 2,172 bp were detected that differed from the corresponding pBC16 sequences by 1 and 4 bp, respectively (Fig. 1). Between these two pBC16-homologous segments, a stretch of 1,589 bp, including the *dfrK* gene, was detected. The *dfrK*-flanking regions did not show homology to sequences deposited in the databases and did not contain additional reading frames for proteins of known function. Exactly at the junctions between pBC16-homologous and -nonhomologous sequences, direct duplications of 21 bp, which showed a single mismatch, were detected (Fig. 1). Since this 21-bp sequence also was present in pBC16, it might have served for the integration of the *dfrK* region into a pBC16-like plasmid. The physical linkage between *dfrK* and *tet(L)* allows the maintenance of a plasmid like pKKS2187 under selective pressure by either tetracyclines or trimethoprim, both of which are widely used in veterinary medicine.

**Nucleotide sequence accession number.** The sequence of the 7,045-bp segment of plasmid pKKS2187 has been deposited in the EMBL database under accession number FM207105.

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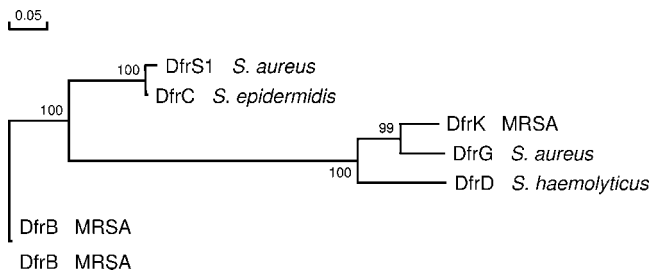


FIG. 2. Phylogenetic tree of the staphylococcal DfrS1 (14), DfrD (1, 3), DfrG (17), and DfrK (this study) proteins, which are involved in trimethoprim resistance, and the DfrB (6, 7) and DfrC (2) proteins, which represent trimethoprim-sensitive dihydrofolate reductases. Branch lengths are scaled according to amino acid exchanges observed in a multisequence alignment. The numbers at the major branch points refer to the percentage of times that a particular node was found in 10,000 bootstrap replications.

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