

Identification of a Plasmid-Borne Resistance Gene Cluster Comprising the Resistance Genes *erm*(T), *dfrK*, and *tet*(L) in a Porcine Methicillin-Resistant *Staphylococcus aureus* ST398 Strain[∇]

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A novel plasmid-borne resistance gene cluster comprising the genes *erm*(T) for macrolide-lincosamide-streptogramin B resistance, *dfrK* for trimethoprim resistance, and *tet*(L) for tetracycline resistance was identified in a porcine methicillin-resistant *Staphylococcus aureus* sequence type 398 (ST398) strain. This *erm*(T)-*dfrK*-*tet*(L) region was flanked by copies of the novel IS element ISSau10. The *erm*(T) region resembled that of *Streptococcus pyogenes* plasmid pRW35. The *erm*(T) gene of pKKS25 was expressed constitutively due to a 57-bp deletion in the *erm*(T) translational attenuator.

In recent years, methicillin (meticillin)-resistant *Staphylococcus aureus* (MRSA) strains of sequence type 398 (ST398) have gained particular attention as a new clonal lineage associated with livestock (7, 22, 23, 28, 30). MRSA ST398 strains have been mainly found in pigs (7, 22, 23) but are also able to colonize and cause infections in other animal species and humans (13, 21, 28, 30). Recent studies have shown that virtually all MRSA ST398 strains are resistant to β -lactam antibiotics and tetracyclines (7, 13, 21, 22, 28) but vary in their patterns of resistance to other antimicrobial agents, including macrolides, lincosamides, and/or streptogramins (MLS). Resistance to MLS antibiotics in staphylococci is mediated by a number of different resistance genes which specify either target site-modifying enzymes, efflux pumps, or drug-inactivating enzymes (16). The latest update of MLS resistance genes (<http://faculty.washington.edu/marilynr>) listed the rRNA methylase genes *erm*(A), *erm*(B), *erm*(C), *erm*(F), *erm*(G), *erm*(Q), *erm*(Y), and *erm*(33) as occurring in staphylococci. Among these, *erm*(A), *erm*(B), and *erm*(C) have been found most frequently among staphylococci of animal origin (5, 11, 12). In MRSA ST398, macrolide-lincosamide (ML) resistance has been detected at various levels (7, 17, 21, 22, 28). In the few cases in which the genes responsible for ML resistance have been determined, the rRNA methylase genes *erm*(A), *erm*(B), and/or *erm*(C) have been identified (7, 10, 17, 28). In the present study, we investigated a porcine MRSA ST398 strain for the presence of the ML resistance gene, its location on a plasmid, and its association with other resistance genes.

MRSA ST398 strain 25 was obtained in 2008 from the nasal swab of a young sow in the GERM-Vet monitoring program. MIC determination by broth microdilution following the recommendations and using the clinical breakpoints given in CLSI documents M31-A3 (1) and M100-S19 (2) confirmed that this strain was resistant to β -lactam antibiotics, tetracycline, macrolides, lincosamides, and trimethoprim (Table 1).

PCR analysis (9, 14, 15, 18) confirmed the presence of the genes *mecA* and *blaZ* for β -lactam resistance, the tetracycline resistance genes *tet*(K), *tet*(L), and *tet*(M), and the novel trimethoprim resistance gene *dfrK*. PCR screening for the most predominant ML resistance genes in staphylococci (11, 12) did not yield positive results. To investigate whether ML resistance was plasmid borne, plasmids were prepared and transformed into *S. aureus* RN4220 with subsequent selection on medium containing erythromycin (15 μ g/ml) (8–10). A plasmid of ca. 40 kb, designated pKKS25, was identified and shown to confer not only combined resistance to macrolides and lincosamides but also tetracycline resistance via *tet*(L) and trimethoprim resistance via *dfrK*. Plasmid pKKS25 was subjected to restriction analysis with EcoRI, HindIII, and BglII, and the resulting fragments were cloned into the plasmid vector pBlueScript II SK+. Recombinant plasmids were transformed into macrolide-susceptible *Escherichia coli* strain AS19, and transformants were selected on erythromycin-containing Luria-Bertani agar (15 μ g/ml).

A 6,229-bp segment of pKKS25 was sequenced by primer walking on both strands from overlapping clones. A schematic representation of the genes found in this segment is shown in Fig. 1. ML resistance proved to be due to the rRNA methylase gene *erm*(T), which has previously been found in lactobacilli (6, 19, 27), streptococci (3, 4, 20, 29), and enterococci (4) but has not yet been identified in staphylococci. The *erm*(T) gene coded for a 244-amino-acid (aa) protein which was indistinguishable from the chromosomal Erm(T) protein of *Streptococcus pasteurianus* (20) but differed by 2 aa from the plasmid-encoded Erm(T) proteins of *Lactobacillus* sp. (27), *Lactobacillus reuteri* (19), and *Streptococcus pyogenes* (29). A detailed analysis of the *erm*(T) gene region of plasmid pKKS25 revealed identity to the *erm*(T) region of plasmid pRW35 from *S. pyogenes* in a stretch of 123 bp upstream and 139 bp downstream of *erm*(T) (Fig. 1). Previous studies identified a complete translational attenuator immediately upstream of the *erm*(T) gene which consisted of two pairs of inverted-repeat sequences of 12 bp each and a reading frame for a regulatory peptide of 19 aa (19, 20, 29). This translational attenuator closely resembled the

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TABLE 1. Comparative analysis of the MICs of original pKKS25-carrying MRSA strain 25, *S. aureus* RN4220, and the *S. aureus* RN4220 transformant carrying plasmid pKKS25

Bacterial strain	Resistance genes	MIC ($\mu\text{g/ml}$) ^a																	
		PEN	AMP	OXA	ERY	TYL	CLI	TMP	TET	GEN	APR	SPE	CHL	ENR	TIA	VAL	LZD	VAN	Q-D
<i>S. aureus</i> RN4220		≤ 0.03	0.06	0.06	0.25	1	≤ 0.12	0.5	0.06	0.25	1	64	4	0.25	0.12	0.03	0.5	1	0.25
<i>S. aureus</i> RN4220/ pKKS25	<i>erm(T)</i> , <i>dfrK</i> , <i>tet(L)</i>	≤ 0.03	0.06	0.06	≥ 128	≥ 64	≥ 64	≥ 128	32	0.25	1	64	4	0.25	0.12	0.03	0.5	1	0.5
MRSA 25	<i>erm(T)</i> , ^b <i>dfrK</i> , ^b <i>tet(L)</i> , ^b <i>tet(K)</i> , <i>tet(M)</i> , <i>mecA</i> , <i>blaZ</i>	16	16	8	≥ 128	≥ 64	≥ 128	128	0.5	0.5	4	8	0.25	0.5	0.03	0.5	1	1	1

^a Abbreviations: PEN, penicillin G; AMP, ampicillin; OXA, oxacillin; ERY, erythromycin; TYL, tylosin tartrate; CLI, clindamycin; TMP, trimethoprim; TET, tetracycline; GEN, gentamicin; APR, apramycin; SPE, spectinomycin; CHL, chloramphenicol; ENR, enrofloxacin; TIA, tiamulin; VAL, valnemulin; LZD, linezolid; VAN, vancomycin; Q-D, quinupristin-dalfopristin.
^b Associated with plasmid pKKS25.

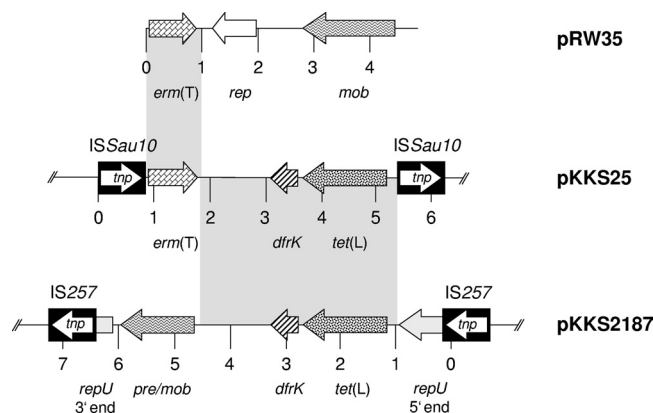


FIG. 1. Comparison of plasmid pKKS25 (accession no. FN390947), identified in the present study, with the *tet(L)*-*dfrK* segment of plasmid pKKS2187 (accession no. FM207105) and *erm(T)*-carrying plasmid pRW35 (accession no. EU192194). The arrows indicate the extents and directions of transcription of the genes *rep*, *repU* (plasmid replication), *tet(L)* (tetracycline resistance), *dfrK* (trimethoprim resistance), *pre/mob* (plasmid recombination/mobilization), and *erm(T)* (combined resistance to macrolides and lincosamides). The IS257 elements in the map of pKKS2187 and the ISSau10 elements in the map of pKKS25 are shown as black boxes with the white arrow indicating the transposase gene *tnp*. The regions with >99% homology between pKKS25 and plasmids pKKS2187 and pRW35 are marked by gray shading.

erm(C)-associated translational attenuator in staphylococci. A complete translational attenuator is required for inducible *erm* gene expression, while constitutive *erm* gene expression is often based on deletions or duplications in this regulatory region (24). Strains carrying constitutively expressed *erm* genes are resistant to noninducers such as lincosamides and 16-membered macrolides (24, 26). A truncated attenuator had been identified upstream of the *erm(T)* gene of plasmid p121BS from a tylosin-resistant *Lactobacillus* sp. (27). Plasmid pKKS25 was isolated from an MRSA strain that exhibited a resistance phenotype indicative of constitutive *erm* gene expression, namely, resistance to clindamycin and tylosin. Analysis of the pKKS25-associated translational attenuator revealed a 57-bp deletion which comprised the initial 40 bp of the reading frame for the 19-aa regulatory peptide, including the upstream part with the ribosome binding site (Fig. 2a). Based on the knowledge of structural changes in the translational attenuators of *erm(C)* genes, limited areas of homology are sufficient to allow recombinational events that account for deletions (26). Hence, it is most likely that the observed 57-bp deletion was also due to such a recombination (Fig. 2b). The remaining part of the resistance gene region of plasmid pKKS25, including the *dfrK* and *tet(L)* genes, corresponded closely (>99% nucleotide sequence identity) to that of plasmid pKKS2187 (9).

This *erm(T)*-*dfrK*-*tet(L)* resistance gene region was flanked by two copies of the novel insertion sequence ISSau10 oriented in the same direction (Fig. 1). This insertion sequence consisted of 793 bp and showed 87 to 88% identity to IS431 or IS257. The 224-aa transposase of ISSau10 exhibited 92 and 93% amino acid identity to the transposases of IS431 and IS257, respectively. The novel IS element carried 16-bp perfect inverted repeats (5'-GGTTCTGTTGCAAAGT-3') at both termini. IS257 elements were shown to be involved in the integration of *tet(L)*-*dfrK*-carrying, as well as *tet(K)*-carrying,

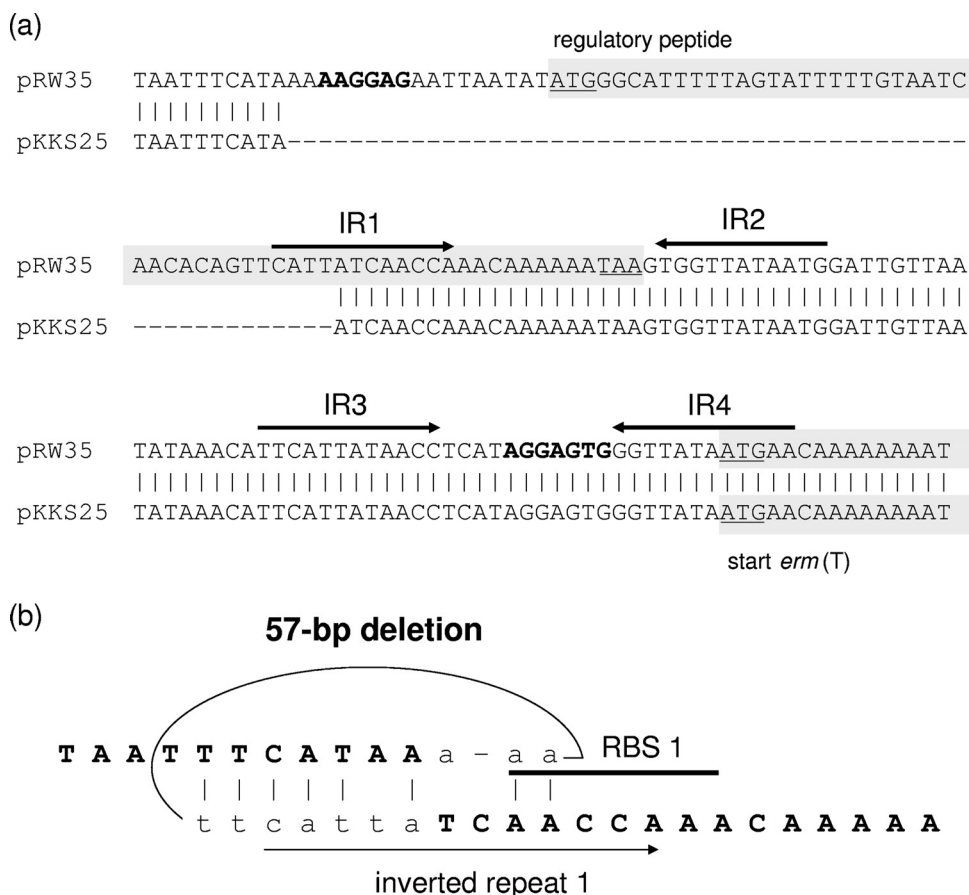


FIG. 2. (a) Comparison of the complete *erm*(T) regulatory region of pRW35 with the truncated region of pKKS25. The 57-bp deletion in pKKS25 is indicated by dashes. Vertical bars indicate bases that are identical in the two sequences. The two pairs of inverted-repeat sequences, IR1-IR2 and IR3-IR4, are marked by arrows. The reading frames for the 19-aa regulatory peptide and the 5'-terminal part of the *erm*(T) gene are indicated by gray shading; translational start and stop codons are underlined. Ribosomal binding sites RBS 1 and RBS 2 are displayed in boldface letters. (b) Possible site for recombination in the *erm*(T) translational attenuator of plasmid pKKS25 leading to the observed 57-bp deletion. The sequence determined in the pKKS25-associated translational attenuator is displayed in boldface capital letters. The lowercase letters represent the beginning and end of the deleted sequence. Important structural elements with respect to panel a, such as the ribosomal binding site RBS 1 and the IR1 sequence, are indicated. Vertical bars mark identical bases in the sequences involved in the recombinational events.

plasmids into larger plasmids (9, 25), and *IS1216V*-like elements were shown to be involved in the integration of the *erm*(T) gene into the chromosome of *S. pasteurianus* (20). It is likely that the novel *ISSau10* insertion sequences played a role in the formation of the resistance gene cluster detected on plasmid pKKS25.

In conclusion, this is the first description of the gene *erm*(T) in staphylococci. Taking into account that tetracyclines, trimethoprim, and macrolides are among the most frequently sold antimicrobial agents in veterinary medicine worldwide, a gene cluster consisting of *erm*(T), *dfrK*, and *tet*(L) offers excellent options for coselection under the selective pressure imposed by the use of any of these antimicrobial agents. Moreover, the finding of *erm*(T) in an MRSA ST398 strain points toward the gene acquisition capacities of such strains and possible partners for gene exchange processes beyond the genus *Staphylococcus*.

Nucleotide sequence accession number. The sequence of the resistance gene region of plasmid pKKS25 has been deposited in the EMBL database under accession number FN390947.

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