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Novel *erm*(T)-Carrying Multiresistance Plasmids from Porcine and Human Isolates of Methicillin-Resistant *Staphylococcus aureus* ST398 That Also Harbor Cadmium and Copper Resistance Determinants

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This study describes three novel *erm*(T)-carrying multiresistance plasmids that also harbor cadmium and copper resistance determinants. The plasmids, designated pUR1902, pUR2940, and pUR2941, were obtained from porcine and human methicillin-resistant *Staphylococcus aureus* (MRSA) of the clonal lineage ST398. In addition to the macrolide-lincosamide-streptogramin B (MLS_B) resistance gene *erm*(T), all three plasmids also carry the tetracycline resistance gene *tet*(L). Furthermore, plasmid pUR2940 harbors the trimethoprim resistance gene *dfrK* and the MLS_B resistance gene *erm*(C), while plasmids pUR1902 and pUR2941 possess the kanamycin/neomycin resistance gene *aadD*. Sequence analysis of approximately 18.1 kb of the *erm*(T)-flanking region from pUR1902, 20.0 kb from pUR2940, and 20.8 kb from pUR2941 revealed the presence of several copies of the recently described insertion sequence IS*Sau10*, which is probably involved in the evolution of the respective plasmids. All plasmids carried a functional cadmium resistance operon with the genes *cadD* and *cadX*, in addition to the multicopper oxidase gene *mco* and the ATPase copper transport gene *copA*, which are involved in copper resistance. The comparative analysis of *S. aureus* RN4220 and the three *S. aureus* RN4220 transformants carrying plasmid pUR1902, pUR2940, or pUR2941 revealed an 8-fold increase in $CdSO_4$ and a 2-fold increase in $CuSO_4$ MICs. The emergence of multidrug resistance plasmids that also carry heavy metal resistance genes is alarming and requires further surveillance. The colocalization of antimicrobial resistance genes and genes that confer resistance to heavy metals may facilitate their persistence, coselection, and dissemination.

Studies of the resistance genes present in livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) of multilocus sequence type (ST) 398 identified a certain heterogeneity of genes encoding the same resistance phenotype (1). This was particularly evident for genes conferring combined resistance to macrolides, lincosamides, and streptogramin B antibiotics (MLS_B) (2–8). The major mechanism of resistance to MLS_B is the methylation of the adenine at position A2058 in domain V of 23S rRNA. So far, at least four rRNA methylase genes, *erm*(A), *erm*(B), *erm*(C), and *erm*(T), have been described in LA-MRSA ST398 (1). Among them, the *erm*(T) gene, although initially reported in other Gram-positive bacteria, such as lactobacilli, streptococci, and enterococci (9–11), has been described for the first time in staphylococci on plasmid pKKS25 in a porcine LA-MRSA ST398 strain (12). This gene has also been detected in LA-MRSA ST398 strains from cattle (3) and food/food products of poultry origin (4). Its presence among MRSA ST398 of human origin has also been described (8). A study of *erm* genes in livestock manure and manure management systems found *erm*(T) at a high frequency in bovine and swine manure (13). Whenever *erm*(T) was detected in MRSA, the corresponding strains showed constitutive resistance to clindamycin, implying the presence of structural alterations in the *erm*(T)-associated translational attenuator (5, 8, 12).

Recent studies have also revealed the presence of the *erm*(T) gene among methicillin-susceptible *S. aureus* (MSSA) ST398 strains (usually of *spa* type t571) in humans who had no contact with livestock (14–18), and in some cases they were involved in serious infections in humans (19–21). In these isolates, the *erm*(T) genes have been shown to confer an inducible clindamycin resistance phenotype and to be located in the chromosomal DNA (15, 16). Very recently, the first *erm*(T)-carrying resistance plasmid, namely, pUR3912, from an MSSA isolate of human origin was described (22). This plasmid carried a functionally active cadmium resistance operon (*cadDX*) and represented the first *erm*(T)-carrying plasmid that also harbored heavy metal resistance determinants. Despite the fact that cadmium is a highly toxic metal that is neither used in agriculture nor found in the community or in the hospital sector, the presence of cadmium resistance determinants on staphylococcal plasmids has been described before (23, 24). In contrast, copper and zinc compounds are commonly used as feed supplements in livestock (25), and zinc resistance has been recently identified as part of type V SCC*mec* cassettes, which are commonly found in LA-MRSA ST398 (26). Thus, it has been assumed that zinc resistance plays a role in the coselection and emergence of methicillin resistance in LA-MRSA ST398 of animal origin.

This study describes novel staphylococcal multiresistance plasmids that also carry cadmium and copper resistance determinants. The aim of the present study was to determine the location and the genetic environment of the resistance genes on *erm*(T)-carrying multiresistance plasmids obtained from five LA-MRSA ST398 strains of porcine and human origin recently detected in Spain (5, 8).

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TABLE 1 Comparative analysis of the MICs of the original *erm*(T)-carrying MRSA strains, *S. aureus* RN4220, and *S. aureus* RN4220 transformants carrying the three novel *erm*(T)-positive plasmids[d]

Bacterial strain	Resistance genes detected for:		MIC (μg/ml) of antimicrobial agent[b]											MIC (mM) of metal		
	Antimicrobial agents[a]	Metal compounds	PEN	OXA	ERY	CLI	TIA	TET	GEN	KAN	NEO	SPE	TMP	$CdSO_4$	$CuSO_4$	$ZnCl_2$
RN4220			0.03	0.12	0.5	0.12	0.5	0.5	0.25	4	0.5	64	1	0.015	8	1
C1902[c]	*blaZ*, *mecA*, *erm*(T), *tet*(L), *tet*(M), *aadD*, *aacA-aphD*	*cadD*, *cadX*, *mco*, *copA*	16	≥16	≥64	≥128	1	64	8	≥512	128	128	2	0.25	8	2
RN4220/pUR1902	*erm*(T), *tet*(L), *aadD*	*cadD*, *cadX*, *mco*, *copA*	≤0.015	0.12	≥64	≥128	0.25	64	0.25	32	16	64	0.5	0.125	16	1
C2940	*blaZ*, *mecA*, *erm*(T), *erm*(C), *vga*(A), *tet*(L), *tet*(M), *dfrK*, *aacA-aphD*	*cadD*, *cadX*, Δ*mco*, *copA*, *czrC*	8	8	≥64	≥128	≥128	64	128	≥512	2	128	≥256	0.5	16	8
RN4220/pUR2940	*erm*(T), *erm*(C), *tet*(L), *dfrK*	*cadD*, *cadX*, Δ*mco*, *copA*	≤0.015	0.06	≥64	≥128	0.5	64	0.25	4	0.5	64	≥256	0.125	8	1
C2941	*blaZ*, *mecA*, *erm*(T), *erm*(C), *tet*(L), *tet*(M), *aadD*	*cadD*, *cadX*, *mco*, *copA*, *czrC*	16	≥16	≥64	≥128	0.5	64	0.5	64	64	≥512	0.25	0.5	16	8
RN4220/pUR2941	*erm*(T), *tet*(L), *aadD*	*cadD*, *cadX*, *mco*, *copA*	≤0.015	0.12	≥64	≥128	0.25	64	0.25	32	32	64	0.5	0.125	16	1

[a] The antimicrobial resistance genes present in the original strains C1902, C2940, and C2941 were described in previous studies (5, 8).
[b] PEN, penicillin; OXA, oxacillin; ERY, erythromycin; CLI, clindamycin; TIA, tiamulin; TET, tetracycline; GEN, gentamicin; KAN, kanamycin; NEO, neomycin; SPE, spectinomycin; TMP, trimethoprim.
[c] Strains C1905 and C1906 showed the same profile to the antimicrobials and metal compounds tested.
[d] Phenotypes considered resistant to the respective antimicrobials and those tentatively considered resistant to the respective metals are indicated by grey shading.

MATERIALS AND METHODS

Bacterial strains investigated and molecular typing. Five *erm*(T)-positive MRSA ST398 strains identified in previous studies were included (5, 8). Strains C1902, C1905, and C1906 were isolated from healthy pigs and presented the *agr* allotype I, *spa* type t011, and SCC*mec* IVa, while strains C2940 and C2941 came from humans with different diseases, showed *agr* allotype I, *spa* type t011, and harbored the SCC*mec* type V (5, 8). All strains were multiresistant (Table 1). To estimate the clonal relatedness of the strains, pulsed-field gel electrophoresis (PFGE) of total DNA after digestion with ApaI (Roche Pharma, Madrid, Spain) was performed by following the HARMONY protocol (27). ApaI fragments were separated for 20 h at 6 V/cm using pulse time ramping from 2 to 5 s (6).

Isolation and transfer of *erm*(T)-carrying plasmids. Plasmids were extracted and purified using a modified alkaline lysis method (12). Obtained plasmids were transformed by protoplast transformation into *S. aureus* RN4220 with subsequent selection on regeneration plates containing erythromycin (15 μg/ml). The presence of the *erm*(T) gene in the transformants was confirmed by a specific PCR (Table 2). The approximate sizes of the transformed plasmids pUR1902, pUR2940, and pUR2941 were calculated as the sum of the fragment sizes obtained after digestion of the plasmids with the restriction endonucleases EcoRI and BglII (Roche) in independent experiments.

Cloning of *erm*(T) and flanking regions from plasmids pUR1902, pUR2940, and pUR2941. Plasmids were digested with EcoRI (for pUR1902 and pUR2941) and by EcoRI and BglII (for pUR2940). The corresponding fragments were cloned into the plasmid vector pBluescript II SK(+) (Stratagene, Amsterdam, The Netherlands), and recombinant plasmids were transformed into *Escherichia coli* JM101. The cloned fragments of interest were sequenced by primer walking on both strands, starting with M13 universal and reverse primers (22). Linkage between sequenced fragments as well as determination of DNA regions that could not be cloned in repeated experiments were performed by PCR mapping. For this, primers were designed from the sequences of already known segments deposited in the GenBank database and the amplicons were sequenced.

Antimicrobial susceptibility testing. MICs for the antimicrobial agents listed in Table 1 were determined for the original strains and their *S. aureus* RN4220 transformants by broth microdilution (28) using custom-made microtiter plates (MCS Diagnostics, Swalmen, The Netherlands). *S. aureus* ATCC 29213 served as a quality control strain.

MIC determinations for cadmium, copper, and zinc compounds and testing of the resistance genes involved. The MIC for cadmium sulfate ($CdSO_4$; Panreac, Barcelona, Spain) was determined by agar dilution in three independent assays on Mueller-Hinton (MH; Becton Dickinson, Madrid, Spain) agar plates, while the MICs for copper sulfate and zinc chloride ($CuSO_4$ and $ZnCl_2$; Scharlau, Barcelona, Spain) were likewise determined on cation-adjusted Mueller-Hinton II (MH-II; Becton Dickinson, Madrid, Spain) agar with the pH of the medium adjusted to 5.5 for $ZnCl_2$ or to 7.4 for $CuSO_4$ (25, 26). For this, the original strains C1902, C1905, C1906, C2940, and C2941, the recipient strain *S. aureus* RN4220, and also the isogenic *S. aureus* RN4220 transformants were used. Concentration ranges for $CdSO_4$ were 0.001 to 2 mM, while those for $CuSO_4$ and $ZnCl_2$ were 0.125 to 128 mM (26). Plates were incubated for 20 h at 37°C under aerobic conditions. The presence of genes responsible for heavy metal resistance (cadmium, copper, zinc) was investigated by PCR and subsequent sequencing of the respective amplicons (Table 2).

Southern blotting for the possible chromosomal localization of *erm*(T) and associated genes. Southern blot analysis of genomic DNA after previous digestion with endonuclease I-CeuI (New England BioLabs, Barcelona, Spain) was performed. Agarose plugs with genomic DNA of strains C1902, C2940, and C2941 were digested with I-CeuI (10 U) for 4 h at 37°C. Fragments were separated in a 1% (wt/vol) PFGE agarose gel (18 h, 6 V/cm, and 5–30 s at 14°C) and I-CeuI PFGE digests were transferred to a nylon membrane. Hybridization with probes for *erm*(T), *cadDX*, *copA*, *mco*, and the 16S rRNA gene and subsequent detection were conducted according to the manufacturer's recommendations (Roche, Madrid, Spain).

Nucleotide sequence accession numbers. The nucleotide sequences of the sequenced parts of plasmids pUR2941 (20,776 bp), pUR1902 (18,126 bp), and pUR2940 (19,957 bp) have been deposited in the EMBL database under accession numbers HF583290, HF583291, and HF583292, respectively.

RESULTS AND DISCUSSION

PFGE profiles, antimicrobial resistance patterns, and plasmid profiles. All five strains shared closely related ApaI PFGE profiles,

TABLE 2 Primers and PCR conditions employed[a]

Gene or region amplified	Primer designation	Primer sequence (5′→3′)	Nucleotide position in published sequence	Amplicon size (bp)	Reference	GenBank accession nos.[b]
erm(T)[c]	ermT_fw	ATTGGTTCAGGGAAAGGTCA	109–128 in *erm*(T)	536	3	HF583290, HF583291, HF583292
	ermT_rv	GCTTGATAAAATTGGTTTTTGGA	622–644 in *erm*(T)			
cadD	cadD-fw2	TGCTAGAGCAAAGACTAGGAAAGA	93–116 in *cadD* (81–104 in *cadD* of pUR3912)	460	This study	HF583290, HF583291, HF583292, HE805623
	cadD-rv	AGCCATAATCCAACGACCAA	533–552 in *cadD* (521–540 in *cadD* of pUR3912)			
cadX	cadX-fw	TGCTTGTGATGTGATCTGTGT	15–35 in *cadX*	213	This study	HF583290, HF583291, HF583292, HE805623
	cadX-rv	TGATGTGAAGTTGAAGCAACAC	206–227 in *cadX*			
Promoter *cadDX*	Pr_cadDX-fw	CTGACGATGCCAGGAAACTT	438–457 upstream of *cadD*	579, 419[d]	This study	HF583290, HF583291, HF583292, HE805623
	Pr_cadDX(pUR3912)-fw	AGTAAGGGTGCAGTGCCAAT	290–309 upstream of *cadD* of pUR3912			
	Pr_cadDX-rv2	CGATATTCTTTCCTAGTCTTTGCTC	98–122 in *cadD* (86–110 in *cadD* of pUR3912)			
copA	copA-fw	CATGCTTTAGGCTTGGCAAT	931–950 in *copA*	662	This study	HF583290, HF583291, HF583292
	copA-rv	TCTTCTGGCATGAGTTGTGC	1573–1592 in *copA*			
mco	mco-fw	TCCCTCCCCAAATACAGCTA	537–556 in *mco*	699	This study	HF583290, HF583291, HF583292
	mco-rv	GTTCCGTGGATATGGAATGG	1216–1235 in *mco*			
czrC	czrC-fw	TAGCCACGATCATAGTCATG	48–67 in *czrC*	655	26	JCSC6944
	czrC-rv	ATCCTTGTTTTCCTTAGTGACTT	680–702 in *crzC*			

[a] Primers and PCR conditions employed in this study to detect genetic determinants for resistance to the different metal compounds detected in the three novel plasmids described in the study and those detected in the recently described pUR3912, as well as those for the MLS_B resistance gene *erm*(T). The PCRs were performed using BioTaq DNA polymerase (Bioline; Cultek, Madrid, Spain) and the following conditions: initial cycle of 3 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 56°C [45°C for the *erm*(T) gene], and 1 min at 72°C, and with a final step of 5 min at 72°C.

[b] Published sequences from which primers were designed and/or coordinates have been established.

[c] It should be noted that this pair of primers could not properly amplify the *erm*(T) gene of a set of *erm*(T)-positive MSSA isolates of the sublineage ST398, which seems to be associated with humans.

[d] Amplicon size obtained using the combination of Pr_cadDX(pUR3912)-fw and Pr_cadDX-rv2.

with the porcine strains C1902, C1905, and C1906 exhibiting even indistinguishable fragment patterns (data not shown). MIC values determined for the original strains and their *S. aureus* RN4220 transformants are shown in Table 1. In addition to the previously described data (5, 8), strain C2940 also revealed resistance to tiamulin (MIC, ≥128 μg/ml), due to the presence of the *vga*(A) gene, and strain C2941 showed elevated MIC values for spectinomycin (≥512 μg/ml), although it was negative for the presence of the spectinomycin resistance gene *spc*.

On the basis of plasmid sizes, EcoRI and BglII restriction patterns, and antimicrobial resistance profiles, three different types of *erm*(T)-carrying plasmids were distinguished. Strains C1902, C1905, and C1906 of porcine origin carried plasmids of ~22 kb that showed the same restriction patterns. As a representative, plasmid pUR1902 from strain C1902 was included in a further analysis; strain C2940 harbored the ~25-kb plasmid pUR2940 and strain C2941 harbored the ~33-kb plasmid pUR2941. MIC testing and PCR analysis of the transformants revealed that, in addition to MLS_B resistance due to the *erm*(T) gene, transformants carrying plasmid pUR1902, pUR2940, or pUR2941 were tetracycline resistant and carried the *tet*(L) gene. In addition, plasmids pUR1902 and pUR2941 conferred resistance to kanamycin/neomycin and had an *aadD* gene, while plasmid pUR2940 conferred trimethoprim resistance via the gene *dfrK*. Moreover, plasmid pUR2940 harbored the additional MLS_B resistance gene *erm*(C) (Table 1).

Analysis of the novel pUR1902, pUR2940, and pUR2941 plasmids. Figure 1 shows maps of the sequenced parts of the three novel multiresistance plasmids in comparison to previously known staphylococcal plasmids that share significant structural similarities. All three plasmids, pUR1902, pUR2940, and pUR2941, had in common the resistance genes *erm*(T) and *tet*(L), a plasmid mobilization gene, *mob*, a 3′-truncated replication gene, *rep49*, the cadmium resistance *cadDX* operon, a *repA*-like gene (70.9% identity to *repA* of pUB101), and two copies of the insertion sequence IS*Sau10* (Fig. 1).

Plasmid pUR2940 exhibited a 5,436-bp resistance region that was 99.9% identical to that of plasmid pKKS25 (12) (Fig. 1). This region comprised the resistance genes *erm*(T), *tet*(L), and the *dfrK*, in addition to one IS*Sau10* copy. A pair of 21-bp imperfect direct duplications (5′-TTCCATTAAC/AGGGCGCGATTG-3′ [the two underlined bases are the ones exchanged in the different sequences]) was found that flanked the 1,590-bp region that comprised the *dfrK* gene (Fig. 1). As previously suggested (29), this sequence might have served for the integration of the *dfrK* region into pUR2940. Immediately downstream of the aforementioned IS*Sau10*, a region delimited by another IS*Sau10* copy was observed. The region between both IS elements was 98.9% identical to the small *erm*(C)-carrying plasmid pNE131 (Fig. 1). The *repL* gene of this small plasmid was interrupted by two IS*Sau10* elements that were located in the same orientation. An 8-bp target site duplication, 5′-GACTGTTC-3′, was detected upstream of the left-hand IS*Sau10* and downstream of the right-hand IS*Sau10* (Fig. 1). The *erm*(C) gene was located between the two *repL* segments. This region exhibited the typical structural characteristics for the integration of a small plasmid into a larger one via ISs (24).

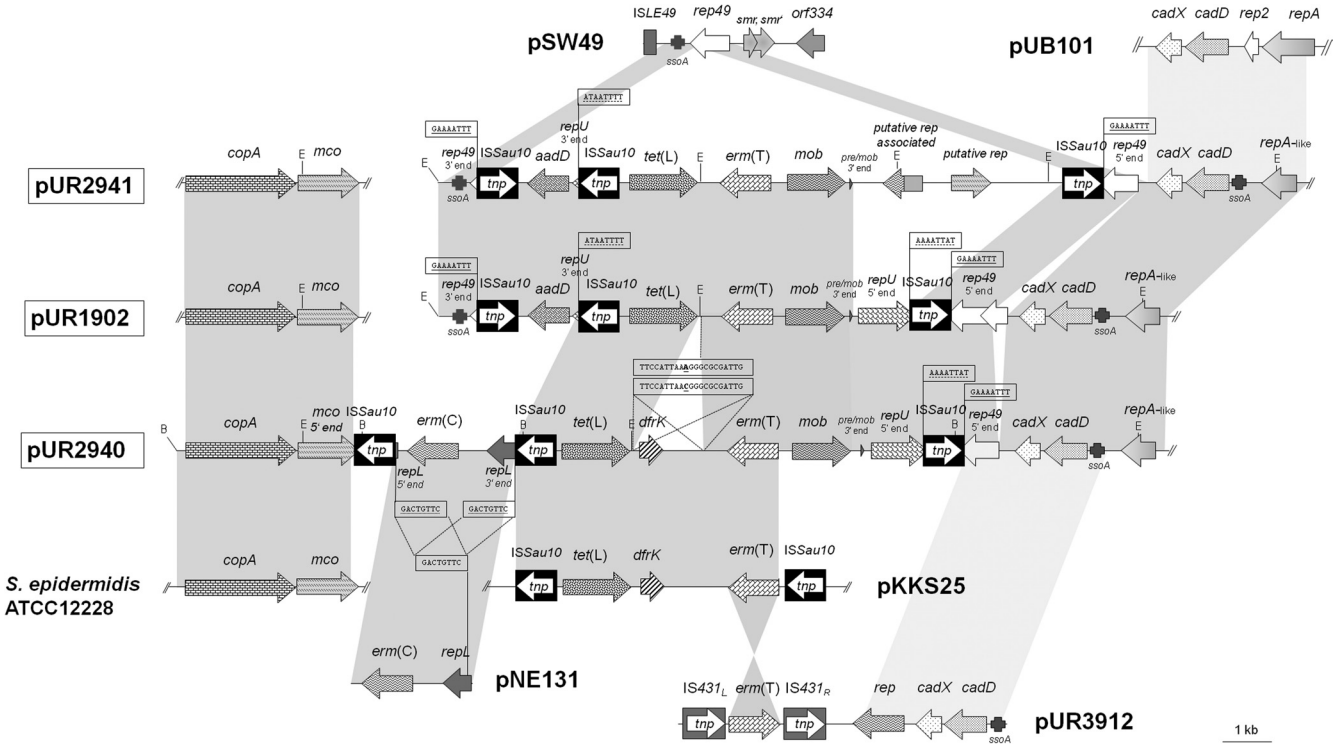

FIG 1 Comparative schematic presentation of the sequenced segments of the three novel *erm*(T)-carrying plasmids (pUR2941 [accession number HF583290], pUR1902 [HF583291], and pUR2940 [HF583292]) described in this study and the previously reported plasmids pSW49 (AM040730), pUB101 (AY373761), pKKS25 (FN390947), pNE131 (M12730), and pUR3912 (HE805623), as well as a chromosomal fragment of *S. epidermidis* ATCC 12228 (AE015929). The arrows indicate the extents and directions of transcription of antimicrobial [*erm*(T), *tet*(L), *aadD*, *dfrK*] and metal (*cadD*, *cadX*, *copA*, *mco*) resistance genes as well as genes involved in replication (*rep49*, *repU*, *repL repA-like*, putative *rep-associated*, putative *rep*, *repA*, and *rep2*), mobilization (*mob*), and others (ISLE49, *smr*, *smr′*, *orf334*). The 5′ and 3′ ends of the truncated *rep49*, *repU*, and *repL* genes and the 5′ end of the truncated *mco* gene in pUR2940 are likewise displayed. The different SSO regions detected are indicated as *ssoA*. The IS*Sau10* and IS*431* copies are shown as black or gray boxes with a white arrow indicating the transposase gene *tnp*. The 8-bp direct target site duplications at the extremes of IS*Sau10* are shown in boxes and underlined. The 21-bp sequences of the integration site of the *dfrK*-carrying region are also boxed. The regions of >90% homology are shown in dark gray, while those between 80 and 90% similarity are displayed in light gray. The EcoRI (E) and BglII (B) cleavage sites are indicated. A size scale in kilobases is displayed in the lower right corner.

Immediately downstream of this segment, the copper resistance genes *copA* and Δ*mco*, whose 3′ end was truncated by IS*Sau10*, were detected (Fig. 1).

Two discontinuous regions were sequenced in plasmids pUR1902 and pUR2941: (i) *erm*(T)-carrying segments of 14,626 bp and 17,276 bp, respectively, obtained from several contiguous EcoRI fragments, and (ii) a *copA*- and *mco*-carrying segment of 3,500 bp obtained by PCR mapping (Fig. 1). Despite extensive attempts, these two regions could not be joined by PCR mapping. Except for a single mismatch, the *copA-mco* segments were identical to that of pUR2940 but harbored the complete *mco* gene. At the left terminus of the *erm*(T)-flanking region of both plasmids, a region of 2,702 bp was detected that was absent in pUR2940. This small fragment carried another IS*Sau10* copy, whose upstream region showed the highest identity to a segment of the *Staphylococcus warneri* small plasmid pSW49, which includes a single-strand origin of replication, *ssoA*, and the 3′ end of *rep49* (Fig. 1). The region downstream of the IS*Sau10* contained the *aadD* gene and the 3′ end of *repU*, which were identical to the corresponding regions of plasmid pKKS825 from MRSA ST398 (30) (Fig. 1). In addition, plasmid pUR2941 exhibited a unique segment of 4,140 bp in which two putative *rep* genes of 744 bp and 726 bp were detected.

Neither the *erm*(T) gene nor the cadmium nor copper resistance determinants (*cadD*, *cadX*, *copA*, or *mco*) detected were present on any of the chromosomal DNA fragments obtained after I-CeuI digestion of C1902, C2940, and C2941 genomic DNA. In contrast, the 16S rRNA gene probe yielded the expected positive hybridization results for all chromosomal DNA bands visualized in all three strains (data not shown).

Role of IS elements in the development of novel multiresistance plasmids. IS*Sau10*-related IS*257* and IS*431* have been shown to be responsible for the cointegration of small plasmids within larger plasmids or within the chromosomal DNA (24, 31, 32). The different copies of IS*Sau10* disrupted diverse *rep* genes of small plasmids as well as the *mco* gene of pUR2940. It has been suggested that the truncation of the *rep* genes in the cointegration process of different plasmids is important for the maintenance of the replication system of the original replicon (24). In addition, the presence of two IS*Sau10* copies in the same orientation with the typical 8-bp direct target site duplications at both ends and bracketing a sequence that closely resembles a small *erm*(C)-carrying plasmid in pUR2940 strongly suggests that its integration was IS*Sau10* mediated. IS elements play an important role in the evolution of multidrug resistance plasmids because of their capacity to integrate, split, and undergo homologous recombination with related IS elements. The presence of several copies of the recently described IS*Sau10* element in the

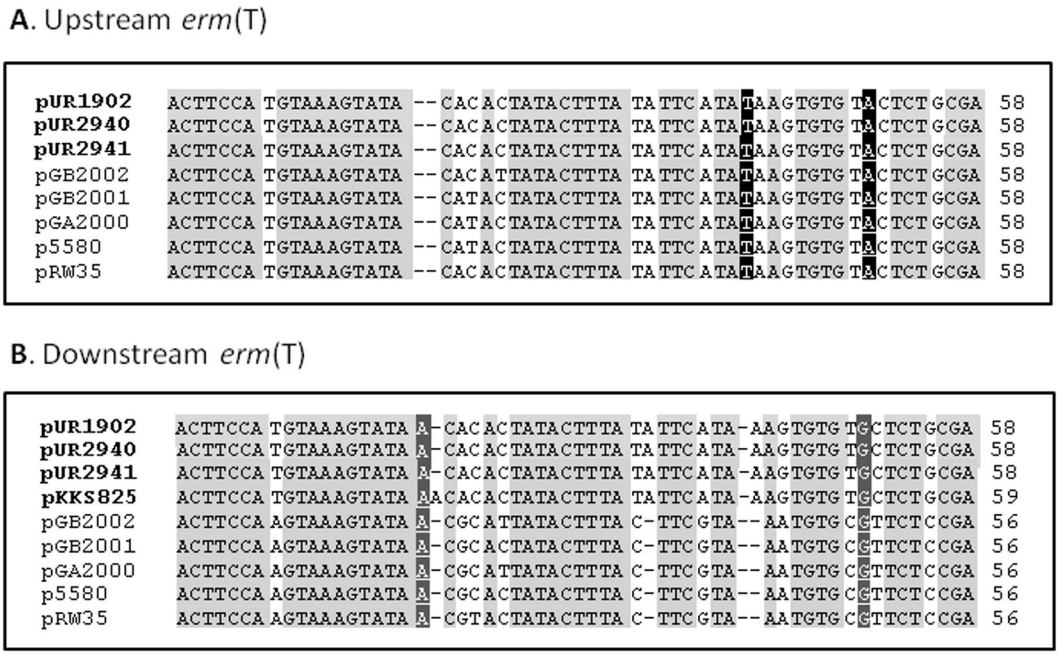

FIG 2 Sequence alignment of the homologous regions (56 to 59 bp) located upstream (A) and downstream (B) of the *erm*(T) gene in the three novel MRSA ST398 plasmids (pUR1902, pUR2940, and pUR2941), plasmid pGB2002 (*Streptococcus agalactiae*), pGB2001 (*Streptococcus agalactiae*), pGA2000 (*Streptococcus pyogenes*), p5580 (*Streptococcus dysgalactiae*), pRW35 (*Streptococcus pyogenes*), and a possible related precursor, pKKS825 (MRSA ST398) potentially used for the integration of an *erm*(T)-containing segment into the hypothetical pUR2940 *erm*(T)-free precursor. Displayed are nucleotides (nt) at the following up- and downstream positions of the *erm*(T) gene, respectively: nt 339 to 396 and 142 to 199 in pUR2940 (accession number HF583292); nt 524 to 581 and 142 to 197 in pRW35 (EU192194); nt 394 to 451 and 142 to 197 in pGB2002 (JF308629); nt 393 to 450 and 142 to 197 in pGB2001 (JF308630), pGA2000 (JF308631), and p5580 (HE862394); nt 1282 to 1340 downstream of the *dfrK* gene in pKKS825 (FN377602). Nucleotides in faint gray are those identical in all sequences, nucleotides in black show the identical bases specific for the upstream segments, and those in dark grey show those identical in all downstream regions.

sequenced regions of all three plasmids suggests that these IS*Sau10* copies play a role in the mosaic structure of plasmids pUR1902, pUR2940, and pUR2941.

Comparative analysis of the *erm*(T) gene and its immediate upstream and downstream regions. Comparative analysis of the complete 735-bp *erm*(T) genes with those deposited in the GenBank/EMBL databases revealed that the *erm*(T) genes of plasmids pUR2940 and pUR2941 were identical to those of streptococcal plasmids and to that of plasmid pKKS25 of MRSA ST398. In contrast, the *erm*(T) gene present in plasmid pUR1902 showed a unique sequence that differed from the aforementioned *erm*(T) genes by a single nucleotide (A416G), resulting in the amino acid change Asn139Ser. A truncated translational attenuator region of *erm*(T) in the three novel plasmids was observed and showed the same 57-bp deletion as found on plasmid pKKS25 (12). Consequently, the three novel plasmids conferred constitutive clindamycin resistance.

Comparison of the up- and downstream sequences of the *erm*(T) genes identified a common region of ~1.4 kb, including the *erm*(T) gene, that was embedded in largely nonhomologous segments in the *erm*(T)-carrying streptococcal plasmids and in the plasmids described in this study. Exactly at the junctions between these homologous and nonhomologous segments, imperfect direct repeats of 56 to 58 bp were detected. These imperfect direct repeats were also present up- (396 bp) and downstream (199 bp) of the *erm*(T) gene in the three novel plasmids (Fig. 2). It is likely that these imperfect direct repeats have played a role in the acquisition of *erm*(T) via recombination by *erm*(T)-free precursors of these plasmids. Interestingly, these regions were not present in the corresponding parts of pUR3912 or strain ST398NM01, what may suggest an alternative *erm*(T) acquisition pathway or subsequent divergence of such regions. Analysis of the *erm*(T)-lacking MRSA ST398 plasmid pKKS825, which also carries the *aadD*, *tet*(L), and *dfrK* genes, identified one such short region of 59 bp (30). This region in pKKS825 differed by only 1 bp from the 58-bp sequence found downstream of *erm*(T) in pUR1902, pUR2940, and pUR2941 (Fig. 2) and may represent a suitable acceptor site for an *erm*(T)-carrying segment.

Susceptibility to cadmium, copper, and zinc compounds and the presence of resistance determinants. Table 1 shows the MIC data for $CdSO_4$, $CuSO_4$, and $ZnCl_2$ of the aforementioned original strains and transformants. An 8-fold increase in the MIC for $CdSO_4$ in *S. aureus* RN4220 transformants carrying pUR1902, pUR2940, or pUR2941 (0.125 mM) was observed compared with the *S. aureus* RN4220 strain (0.015 mM). Original strains and transformants carried the cadmium resistance operon *cadDX*, which consists of the *cadD* gene, encoding a 209-amino-acid (aa) P-type metal efflux pump involved in cadmium resistance, and *cadX*, which codes for a 115-aa protein that serves as a transcriptional regulator.

S. aureus RN4220 transformants carrying pUR1902 or pUR2941 exhibited a 2-fold increase in the MIC values for $CuSO_4$, from 8 mM to 16 mM in comparison to the recipient strain. *S. aureus* RN4220 carrying pUR2940 did not show an increase in the MIC values for $CuSO_4$ in comparison to the recipient strain. The three original strains and their transformants—but not *S. aureus*

RN4220—harbored the copper transport gene *copA*, which codes for a 660-aa P1-type ATPase protein involved in copper resistance, in addition to the multicopper oxidase gene *mco* involved in copper homeostasis. However, accordingly, *S. aureus* RN4220/pUR2940 harbored a truncated Δ*mco* gene, which seemed responsible for the absence of variation in the $CuSO_4$ MIC values, regardless of the presence of the *copA* gene. Although the *copA-mco* genes of pUR1902 and pUR2941 increased the MIC values for copper by only one dilution, it should be noted that this MIC difference is in the high concentration range (16 mM corresponds to >2,000 μg/ml) and as such represents a substantial change in the copper MIC.

Strains C2940 and C2941 revealed 4- to 8-fold-higher MIC values for $ZnCl_2$ (8 mM) in comparison with strain C1902, *S. aureus* RN4220, and the transformants carrying pUR2940, pUR2941, or pUR1902 (1 mM) (Table 1). Accordingly, the presence of the *czrC* gene, which codes for a 644-aa putative cadmium and zinc transporter, was evidenced by PCR and sequencing in the original *S. aureus* strains C2940 and C2941 but neither in C1902 nor in the three *S. aureus* RN4220 transformants.

Simultaneous presence of antimicrobial and metal resistance genes on staphylococcal plasmids. The presence of cadmium resistance determinants in plasmids of *S. aureus* of different lineages is relatively common (23, 24, 33). Likewise, genes involved in copper resistance, especially *copA* and *mco* or variants (*copB* and *copC*), have been detected in different staphylococcal species and other Gram-positive bacteria, such as *Listeria monocytogenes* and *Macrococcus caseolyticus* (33–41). In LA-MRSA ST398, cadmium, copper, and other heavy metal resistance genes had been only detected in the chromosomal DNA, either within the SCC*mec*V$(5C2\&5)_C$, which carries the cadmium/zinc resistance gene *czrC*, or in the novel SCC*mec*IX and -X, which carry cadmium (*cadDX*), copper (*copB-mco*), and arsenic (*arsRBC* and *arsDARBC*) resistance elements (26, 42). The gene cluster *copA-mco* detected in the three plasmids showed the highest percentage of identity to that identified in the chromosomal DNA of *Staphylococcus epidermidis* ATCC 12228 (92.8%), *Staphylococcus haemolyticus* JCSC1435 (92.6%) and the novel SCC*mec*X and SCC*mec*IX elements of MRSA ST398 strains JCSC6945 (91.9%) and JCSC6943 (91.3%), respectively. The current study presents the first report of LA-MRSA ST398 harboring a plasmid that carries cadmium and copper resistance genes.

Elevated concentrations of cadmium in feedstuffs may occur due to the application of sewage sludge or phosphate fertilizers with high levels of cadmium into agricultural soils (43). Even though the accepted maximum levels of cadmium in feedstuffs for livestock are regulated (43), the possibility that such intake might be in part responsible for the selection of cadmium resistance determinants in the present bacterial population of pigs cannot be excluded. In humans, smoking cigarettes is an additional important source of cadmium (44). However, the major route of cadmium intake for the nonsmoking and non-occupationally exposed population is through ingestion of contaminated food (including food of animal origin) and water (43, 45). This exposure might also select for cadmium resistance in transferable elements in the human *S. aureus* population. In contrast, copper and zinc are essential trace elements for animals and humans (46, 47). They are added as feed supplements for livestock, particularly pigs, in great quantities to increase the daily growth rate of piglets (up to 8 to 10 weeks of age), to prevent gastrointestinal infections, and to limit and control cases of postweaning wanting and wasting (26, 28, 46, 47). The presence and maintenance of genetic elements that carry genes for copper and zinc resistance/tolerance in LA-MRSA ST398 are most probably favored by the extensive use of copper and zinc in pig production.

Staphylococcal plasmids that contain antimicrobial and metal resistance genes have been known for a long time. Plasmids of the pI258 type (GenBank accession no. NC_013319) have been disseminated in *S. aureus* ST30 (48) and contain, in addition to the β-lactamase gene *blaZ* and the MLS_B resistance gene *erm*(B), genes conferring resistance to cadmium (*cadA* and *cadC*), arsenic (*arsRBC*), and mercury (*mer* operon), as well as copies of the IS*431* element. In the current study, all three novel plasmids conferred a multidrug resistance phenotype, carried tetracycline and MLS_B resistance genes, as well as kanamycin/neomycin and/or trimethoprim resistance genes. Due to the elevated use of antimicrobial agents, in particular tetracyclines, macrolides, lincosamides, and aminoglycosides (1, 49) in veterinary medicine and food animal production, the colocation of antimicrobial resistance and heavy metal resistance genes on the same plasmid may favor their maintenance and dissemination under the selective pressure imposed by the use of either antimicrobial agents or heavy metals.

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