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Dissemination of a pSCFS3-Like *cfr*-Carrying Plasmid in *Staphylococcus aureus* and *Staphylococcus epidermidis* Clinical Isolates Recovered from Hospitals in Ohio

Rodrigo E. Mendes,[a] Lalitagauri M. Deshpande,[a] Hector F. Bonilla,[b] Stefan Schwarz,[c] Michael D. Huband,[d] Ronald N. Jones,[a] John P. Quinn[d]

JMI Laboratories, North Liberty, Iowa, USA[a]; UPMC Mercy, Pittsburgh, Pennsylvania, USA[b]; Institute of Farm Animal Genetics, Friedrich-Loeffler-Institut, Neustadt-Mariensee, Germany[c]; Pfizer Inc., Groton, Connecticut, USA[d]

Nineteen linezolid-resistant *Staphylococcus epidermidis* and two *Staphylococcus aureus* isolates recovered from two medical institutions in northeast Ohio and an *S. aureus cfr* index strain previously collected in the same facilities during the 2007 SENTRY Antimicrobial Surveillance Program were investigated for the genetic basis of oxazolidinone resistance and the location of *cfr*. *S. aureus* isolates were typed by pulsed-field gel electrophoresis (PFGE), *spa* typing, and multilocus sequence typing (MLST). The location of *cfr* was determined by Southern blotting and hybridization. Plasmid sequencing was performed using the 454 Life Sciences (Roche) GS-FLX DNA platform. The two *S. aureus* isolates showed unique PFGE patterns but were multilocus sequence type 5 (ST5) and *spa* type t002, whereas the *S. aureus* index strain was ST239 and t037. Southern blot and hybridization experiments showed that *cfr* was plasmid located and that the *S. epidermidis* isolates, one of the *S. aureus* isolates, and the *S. aureus* index strain shared an identical *cfr*-carrying plasmid (39.3 kb). Sequencing results confirmed these findings. A 10-kb fragment containing *cfr* showed the highest identity (99.9%) to a 9.5-kb fragment of plasmid pSCFS3 from a bovine *Staphylococcus lentus* isolate from Germany. In addition, these 39.3-kb plasmids from human *S. epidermidis* and *S. aureus* exhibited BglII restriction profiles very similar to that observed for plasmid pSCFS3. The *cfr*-carrying plasmid detected in the remaining *S. aureus* isolate (7.9 kb) was distinct and showed the highest identity to the chromosomal *cfr* integrate found in the chromosomal DNA of a *Proteus vulgaris* isolate from a pig in China.

Linezolid has been widely prescribed to treat serious infections caused by multidrug-resistant (MDR) Gram-positive pathogens since its clinical introduction as the first oxazolidinone in the United States in 2000 and in the United Kingdom in 2001 (1). Linezolid is currently approved by the Food and Drug Administration (FDA) for the treatment of complicated and uncomplicated skin and skin structure infections and for nosocomial and community-acquired pneumonia caused by susceptible organisms. Linezolid also has an FDA indication for the treatment of vancomycin-resistant *Enterococcus faecium* (VRE) infections, including cases with concurrent bacteremia (2).

This oxazolidinone alters protein synthesis via binding to the 50S ribosomal subunit, with more recent data suggesting that this drug binds to the A site of the peptidyl transferase center (PTC) of the bacterial ribosome and interferes with the positioning of aminoacyl-tRNA, resulting in protein synthesis inhibition (3). Although the prevalence of linezolid resistance remains relatively low among Gram-positive surveillance clinical isolates (4, 5), the resistance mechanisms have been extensively characterized. These mechanisms are mostly comprised of mutations in domain V of 23S rRNA, and alterations in the ribosomal proteins L3 and L4 have also been associated with decreased susceptibility (6). A more recent resistance mechanism, namely, *cfr*, has been recognized. The gene *cfr* encodes a methyltransferase that catalyzes the post-transcriptional methylation of nucleotide A2503 (*Escherichia coli* numbering of rRNA) in the 23S rRNA, causing decreased susceptibility to phenicol, lincosamide, oxazolidinone, pleuromutilin, and streptogramin A ($PhLOPS_A$) compounds (6–8).

Initially detected among staphylococcal species recovered from animal and human sources (9–11), the *cfr* gene has now been reported among several Gram-positive isolates, such as *Enterococcus faecalis* (12, 13), *Macrococcus caseolyticus* and *Jeotgalicoccus pinnipedialis* (14), and *Bacillus* spp. (15–17), as well as in isolates of the Gram-negative organisms *E. coli* (18) and *Proteus vulgaris* (19) recovered from several different specimen sources collected from animals. However, recent data have demonstrated the *Bacillales* order as containing the natural reservoirs for the *cfr* genes (20). With a few exceptions, *cfr* has exclusively been associated with mobile elements, such as insertion sequences and mobilization genes carried by a variety of plasmids (6). In this study, 19 linezolid-resistant *Staphylococcus epidermidis* isolates (21) and two *S. aureus* clinical isolates recovered from two medical institutions located in northeast Ohio were included (i) to phenotypically and genotypically characterize these isolates, (ii) to assess the *cfr* location, and (iii) to determine the genetic elements responsible for gene mobilization and/or dissemination.

MATERIALS AND METHODS

Bacterial strains. A total of 22 staphylococci were initially included in this investigation, and isolates comprised 19 *S. epidermidis* (13 from hospital A [April 2008 through May 2009] and 6 from hospital B [October 2006

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Address correspondence to Rodrigo E. Mendes, rodrigo-mendes@jmilabs.com.

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through July 2007]) (21) and 2 *S. aureus* (isolates 1609 and 1900 from hospital B). All *S. aureus* and *S. epidermidis* isolates included were recovered from multiple blood cultures from unique patients and deemed to be clinically significant by local guidelines. In addition, all bloodstream infections occurred after 48 h of hospitalization. *S. epidermidis* isolates were previously reported by Bonilla et al. (21) to be genetically related, and these isolates displayed indistinguishable chromosomal and plasmid profiles (22). Therefore, one representative *S. epidermidis* isolate from each hospital (A [isolate 1243] and B [1519]) was selected for further characterization, unless otherwise specified. Moreover, a *cfr* index strain (strain 737) recovered from the same hospital B complex facilities during the 2007 SENTRY Antimicrobial Surveillance Program was included for comparison purposes (23).

Antimicrobial susceptibility profile. Susceptibility testing was performed by broth microdilution, according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI) (24). *S. aureus* ATCC 29213 and *E. faecalis* ATCC 29212 were tested concurrently for quality assurance purposes (25). In addition, the inoculum density was monitored by colony counts to ensure an adequate number of cells for each testing event. MIC interpretations were applied as described in CLSI document M100-S22 (25), except in the cases of retapamulin and tigecycline. MIC results for retapamulin and tigecycline compounds were interpreted according to the epidemiological cutoff values for *S. aureus* proposed by Traczewski and Brown (i.e., ≤0.5 μg/ml for susceptible, 1 μg/ml for intermediate, and ≥2 μg/ml for resistant) and the FDA (≤0.5 μg/ml for susceptible), respectively (26, 27).

Screening for linezolid resistance mechanisms. Isolates were screened for the presence of *cfr*, as well as mutations in the 23S rRNA and ribosomal proteins (L3 and L4), by PCR and sequencing as previously described (17). Amplicons were sequenced on both strands. Deduced amino acid sequences of ribosomal proteins were compared to those from wild-type *S. epidermidis* ATCC 12228 and *S. aureus* RN4220 using the Lasergene software package (DNAStar, Madison, WI).

Molecular typing. *S. aureus* 1609 and 1900 were subjected to pulsed-field gel electrophoresis (PFGE), as previously described (28). SmaI-digested genomic DNA was resolved in CHEF-DR II (Bio-Rad, Richmond, CA), and PFGE profiles obtained were compared to that of *cfr* index strain 737 and representatives of U.S. clones using GelCompar II software (Applied Math, Kortrijk, Belgium). Percent similarities were identified on a dendrogram derived from the unweighted-pair group method using arithmetic averages and based on Dice coefficients. Band position tolerance and optimization were set at 1.5% and 0.5%, respectively. Isolates showing similarity coefficients of ≥95% were considered genetically indistinguishable (subtype), while those with similarity coefficients between 80.0 and 94.9% were classified as genetically related (type). The *cfr*-carrying *S. aureus* strains 1609 and 1900 were further characterized by *spa* typing and multilocus sequence typing (MLST). The *spa* types were assigned through the Ridom web server (http://www.ridom.de/spaserver/), and MLST alleles and sequence types (ST) were identified using the MLST database (http://www.mlst.net).

***cfr* plasmid transfer, analysis, and sequencing.** Whole genomic DNAs from all 19 *S. epidermidis* isolates and *S. aureus* 1900, 1609, and 737 were prepared in 1% agarose blocks and partially digested with S1 endonuclease. DNA fragments were resolved by PFGE using CHEF-DR II (Bio-Rad), and plasmid band sizes were determined by comparison with bacteriophage λ concatemers (New England BioLabs, Ipswich, MA). Plasmid extractions from *S. epidermidis* 1243 and 1519 and *S. aureus* isolates 1900, 1609, and 737 were obtained by using the plasmid DNA MIDI kit (Qiagen GmbH, Hilden, Germany). Plasmid transfer into wild-type *S. aureus* RN4220 was performed by electroporation using Micropulser (Bio-Rad, Richmond, CA). Probable transformants were selected using retapamulin (0.25 μg/ml). The presence of *cfr* among transformant colonies was confirmed by PCR. Plasmids were digested with restriction enzymes (EarI, NsiI, and AsiI; New England BioLabs) and separated in 1% agarose gels. DNA fragments from agarose gels were transferred to a nylon membrane by Southern blotting. Membranes were hybridized using a digoxigenin-labeled *cfr*-specific probe (Roche Diagnostics GmbH, Mannheim, Germany).

Similar hybridization signal results were obtained for *S. epidermidis* 1243 and 1519 and *S. aureus* 1609 and 737, suggesting that these isolates shared the same *cfr*-carrying plasmid or had closely related plasmids. Therefore, a *cfr* plasmid from *S. epidermidis* 1243 and *S. aureus* 737 and 1900 were selected for further sequencing. Plasmid preparations were subjected to DNA sequencing using the 454 Life Sciences (Roche) GS-FLX DNA platform. DNA assembling and analysis were performed by Lasergene. In addition, *cfr* plasmids from isolates 737, 1609, and 1243 were digested using BglII, and restriction patterns were compared with that of pSCFS3 (9, 29).

Nucleotide sequence accession numbers. The nucleotide sequences of the *cfr*-carrying plasmids described herein have been submitted to the EMBL/GenBank/DDBJ sequence databases and assigned accession numbers KC206006, KC222021, and KC561137.

TABLE 1 Antimicrobial susceptibility profiles for one representative *S. epidermidis* isolate each from hospital A (1243) and B (1519) and *S. aureus* from hospital B (1609 and 1900)

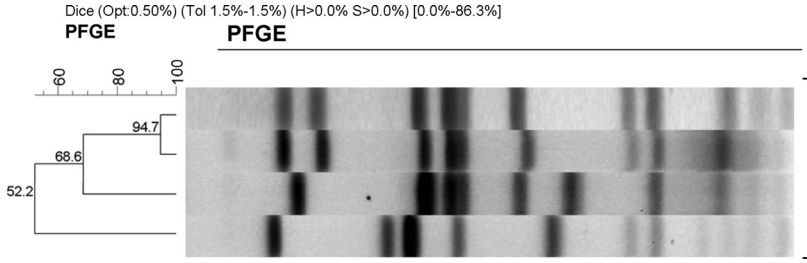
Antimicrobial agent	MIC (μg/ml) (susceptibility category)[a]			
	S. epidermidis		*S. aureus*	
	1243	1519	1609	1900
Linezolid	128 (R)	>128 (R)	16 (R)	8 (R)
Chloramphenicol	>128 (R)	>128 (R)	>128 (R)	64 (R)
Clindamycin	>64 (R)	>64 (R)	>64 (R)	>64 (R)
Virginiamycin	8	16	32	16
Quinupristin-dalfopristin	2 (I)	2 (I)	8 (R)	4 (R)
Retapamulin	>8 (R)	>8 (R)	>8 (R)	>8 (R)
Tiamulin	>64	>64	>64	>64
Tigecycline	0.25 (S)	0.12 (S)	0.25 (S)	0.12 (S)
Tetracycline	0.25 (S)	0.5 (S)	0.5 (S)	0.25 (S)
Vancomycin	2 (S)	2 (S)	1 (S)	1 (S)
Daptomycin	0.25 (S)	0.25 (S)	0.25 (S)	0.25 (S)
Oxacillin	>2 (R)	>2 (R)	>2 (R)	>2 (R)
Ciprofloxacin	>4 (R)	>4 (R)	>4 (R)	>4 (R)
Erythromycin	4 (I)	4 (I)	>4 (R)	>4 (R)
Gentamicin	8 (I)	8 (I)	>8 (R)	≤1 (S)
Trimethoprim-sulfamethoxazole	4 (R)	4 (R)	≤0.5 (S)	≤0.5 (S)

[a] MIC interpretive criteria as published in CLSI document M100-S22, when available (25). Retapamulin MIC results were interpreted according to the microbiological breakpoints proposed by Traczewski and Brown (i.e., ≤0.5 μg/ml for susceptibility, 1 μg/ml for intermediate, and ≥2 μg/ml for resistant) (26). The tigecycline breakpoint for susceptibility was that for *S. aureus* (≤0.5 μg/ml) approved by the FDA (27). S, susceptible; I, intermediate; R, resistant.

RESULTS AND DISCUSSION

Antimicrobial susceptibility and resistance mechanisms. *S. aureus* exhibited linezolid MIC results between 8 and 16 μg/ml, while *S. epidermidis* showed MICs of ≥128 μg/ml (Table 1). Elevated MICs were noted for chloramphenicol (≥64 μg/ml), clindamycin (>64 μg/ml), virginiamycin (8 to 32 μg/ml), quinupristin-dalfopristin (2 to 8 μg/ml), retapamulin (>8 μg/ml), and tiamulin (>64 μg/ml) among all *cfr*-positive staphylococcal isolates (Table 1). All *S. aureus* strains demonstrated wild-type sequences for 23S rRNA and the ribosomal proteins investigated. *S. epidermidis* isolates also had wild-type sequences for 23S rRNA, but L3 amino acid alterations (H146Q, V154L, A157R) were observed in the representative *S. epidermidis* strains, as well as a $_{71}G_{72}$ insertion in the L4 protein (21).

Molecular typing. PFGE analysis revealed that *S. aureus* 1900, 1609, and 737 clustered within three PFGE types. The PFGE pattern of isolate 1900 (type A1) was similar to that of NRS382 (type



Isolate	Hospital	PFGE	*spa*		MLST
			Ridom	Kreiswirth	
NRS382	NA	A	t002	TJMBMDMGMK	5
1900	B	A1	t002	TJMBMDMGMK	5
1609	B	B	t002	TJMBMDMGMK	5
737	B	C	t037	WGKAOMQ	239

FIG 1 PFGE profiles of *cfr*-carrying *S. aureus* 1900 and 1609 (hospital B) compared with 737 (hospital B complex facilities) and a representative strain of the USA100 clone (NRS382). NA, not applicable.

A), a USA100 clone representative (similarity coefficient of 94.7% [Fig. 1]). *S. aureus* 1609 exhibited a unique PFGE pattern, although further typing analysis demonstrated that, similar to USA100 (ST5 and t002), isolates 1900 and 1609 were ST5 (clonal complex 5 [CC5]) and *spa* t002. The index *S. aureus* 737 was ST239 (CC239) and *spa* t037 and had a unique PFGE profile, and these typing results are associated with the Brazilian/Hungarian clone.

Location of *cfr*. All *S. epidermidis* isolates showed identical plasmid profiles during S1 endonuclease experiments (data not shown). Southern blotting and hybridization experiments revealed signals from plasmid bands located slightly below the 48.5-kb ladder, suggesting a plasmid size of approximately 40 kb in the *S. epidermidis* representatives (Fig. 2). Hybridization signals were observed in plasmid bands of similar sizes (~40-kb) from *S. aureus* 1609 and 737, while *S. aureus* 1900 demonstrated signals from three plasmid bands of various sizes (Fig. 2). When plasmid preparations were submitted to restriction digestions prior to Southern blotting and hybridization, the two *S. epidermidis* representatives of each hospital (A and B) and *S. aureus* 737 and 1609 displayed equivalent hybridization signal profiles, while *S. aureus* 1900 showed a distinct pattern with multiple hybridization signals, as for the experiment described above (22). In addition, plasmid DNAs obtained from *S. epidermidis* isolates (1243 and 1519) and *S. aureus* 737, 1609, and 1900 were transferred by electroporation to *S. aureus* RN4220 and results confirmed by PCR. Southern blotting and hybridization experiments performed demonstrated results similar to those observed for the parent isolates, including for the transformant obtained from isolate 1900, which showed multiple hybridization signals (data not shown).

Plasmid sequence analysis. The Southern blotting and hybridization experiments suggested that the *S. epidermidis* isolates and *S. aureus* 1609 and 737 shared a same-size *cfr*-carrying plasmid, which was different from that observed in *S. aureus* 1900. Therefore, these results led to the selection of *cfr* plasmids from one *S. epidermidis* isolate (1243) and two *S. aureus* isolates (737 and 1900) for further DNA sequencing. Sequencing analysis confirmed that the *cfr* plasmids from 1243 and 737 were identical, with no variation in nucleotide sequences. These plasmids, designated pSE1243 and pSA737, had a size of 39.3 kb, and approximately 75% of their DNA sequences showed the highest identity (97%) to pSK73 (GenBank accession no. GQ915269), which originated from an *S. aureus* isolate recovered from a human clinical specimen in 1966 in Sydney, Australia. The pSK73 sequence deposited in GenBank possesses 45 open reading frames (ORF), including resistance determinants [*erm*(A) (macrolide-lincosamide-streptogramin B resistance) and *spc* (spectinomycin resistance gene)] and *tnpA*, *tnpB*, and *tnpC* genes associated with a Tn*554* element. A second, smaller fragment (~10 kb) of pSE1243 and pSA737 harbored the *cfr* gene with a genetic environment identical to that of pSCFS3 (AM086211) and contained Δ*tnpA*, *istAS*, and *istBS* upstream (similar to IS*21*) of *cfr*; Δ*tnpB*, *tnpC*, *orf138*, and *fexA* (similar to Tn*558*) were located downstream (Fig. 3). Compared with the nucleotide sequence available for pSCFS3 (9,497 bp), pSE1243 and pSA737 displayed only 13 nucleotide differences, which consisted of 10 nucleotides within a noncoding region upstream of *istA*, a nucleotide change within *istA* (silent mutation), and one nucleotide mutation each in *istB* and *cfr*, causing glycine-to-arginine and alanine-to-asparagine alterations, respectively. The

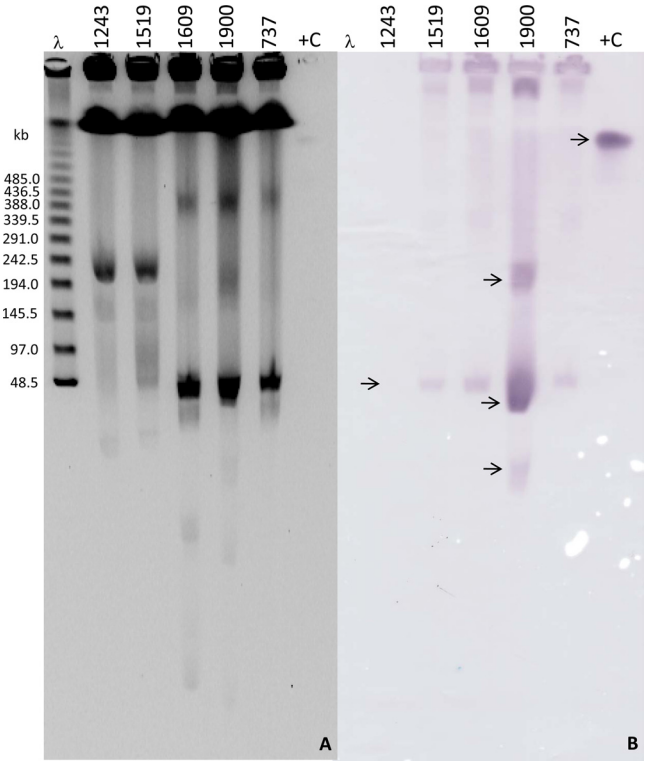


FIG 2 (A) S1 partially digested genomic DNA of representative *S. epidermidis* isolates from each hospital, A and B (1243 and 1519, respectively), and *S. aureus* isolates 1609 and 1900 (from hospital B). "737" represents the *cfr*-carrying *S. aureus* index strain 737 (from hospital B complex facilities) (23). "λ" and "+C" represent lambda ladder PFGE marker and positive control (diluted PCR product), respectively. (B) Hybridization signals (arrows) obtained with a *cfr*-specific probe. Hybridization signals for one of the *S. epidermidis* (1243) are not visible in this particular membrane.

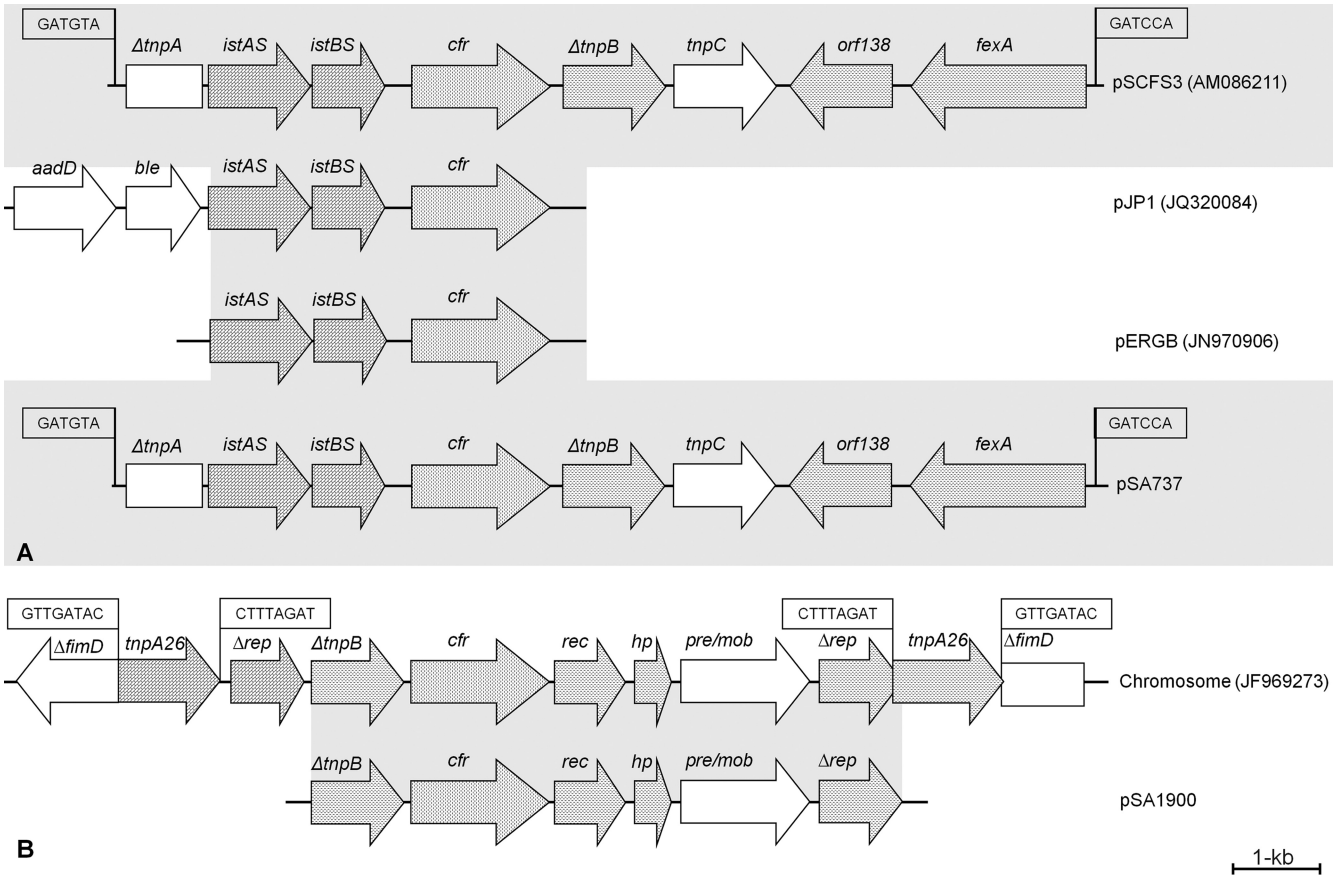


FIG 3 (A) Schematic representation of DNA sequences surrounding *cfr* detected in *S. aureus* 737 and 1609 and *S. epidermidis* 1243, as well as those from pSCFS3 (9), pJP1 (14), and pERGB (35). (B) Representation of DNA sequence surrounding *cfr* detected in *S. aureus* 1900, as well as the *P. vulgaris* chromosomal DNA fragment containing *cfr* (19). The 6- and 8-bp repeats are boxed.

10-kb fragment of plasmid pSCFS3 was reported to have originated by the insertion of a segment containing an IS*21*-like element (IS*21-558*) and the *cfr* gene into Tn*558*, thereby truncating both the *tnpA* and *tnpB* genes of this transposon (9) (Fig. 3). During these events, the resistance genes *erm*(A) and *spc* were deleted and also not observed in pSE1243 or pSA737. These sequence comparisons suggested that pSE1243 and pSA737 (39.3 kb) possess an identical or very similar *cfr*-containing segment compared to pSCFS3, previously reported to have a similar size (35.7 kb). In fact, analysis of plasmid profiles after restriction digestion with BglII demonstrated that these plasmids had similar patterns, except that pSCFS3 had an extra small fragment (2.1 kb) not observed in pSE1243 and pSA737 (data not shown).

The *cfr*-carrying plasmid detected in *S. aureus* 1900 (pSA1900) was 7.9 kb and had five complete (including *cfr*) reading frames, of which four were possibly involved in plasmid replication (Δ*rep*) and mobilization (*rec*, *hp*, and *pre*/*mob*). This context showed complete identity to the flanking regions of the *cfr* gene detected in the chromosome of a *P. vulgaris* isolate from a pig in China (JF969273) (13). Interestingly, hybridization experiments using a *cfr*-specific probe exhibited signals from three distinct plasmids on *S. aureus* 1900 (Fig. 2), which indicates that a smaller and circular plasmid form was sequenced and that likely this fragment was also inserted in other larger plasmids detected in *S. aureus* 1900 (Fig. 2).

More detailed plasmid analysis demonstrated that *cfr* was plasmid located in all isolates, and further sequencing results disclosed that the genetically unrelated *S. aureus* 737 (ST239) and 1609 (ST5) and *S. epidermidis* 1243 shared a closely related *cfr*-carrying plasmid, which was then identified as similar to the previously reported pSCFS3 from bovine *S. lentus* (9). Similar findings have been reported by Kehrenberg et al. (29), who described the detection of pSCFS3 in other lineages (ST9 and ST398) of livestock-associated *S. aureus* recovered from swine during a survey among 367 farms in Germany. pSCFS3-like plasmids seem to be nonconjugative (29); however, they have been shown to be transferable *in vitro* into another staphylococci in this study and elsewhere (9, 29). Although pSCFS3-like plasmids are considered nonconjugative elements, these findings suggest that they can be transferred within staphylococci by other mechanisms. Moreover, it has been reported that nonconjugative staphylococcal plasmids can be mobilized *in vivo* by the copresence of a plasmid or chromosomally located conjugative machinery (30–32). In addition, mechanisms other than conjugation, such as mobilization, transduction, or rolling-circle replication, can also promote plasmid transfer (33).

In contrast, *S. aureus* 1900 carrying pSA1900 had a distinct *cfr* plasmid compared with pSCFS3 or with pSA737 and pSE1243. However, the genetic context of *cfr* observed in pSA1900 was similar to that described for the chromosome of a *P. vulgaris* isolate from China, where *cfr* seemed to have been mobilized by trans-

posases (IS*26* elements) (19). Three hybridization signals were observed during the experiments performed in this study to determine the *cfr* location in *S. aureus* 1900 and the respective transformant. Therefore, these results suggest that the small plasmid containing *cfr* (pSA1900) may have been replicated within the cell by the plasmid replication (Δ*rep*) and mobilization (*rec*, *hp*, and *pre*/*mob*) genes observed or by a transduction mechanism (33). However, it is unclear whether this small *cfr* plasmid can be transferred within staphylococcal species, and further investigations are needed to clarify the mobilization of pSA1900.

In summary, previous studies describing outbreaks caused by Cfr-producing organisms have demonstrated that the vast majority of isolates were clonally related and that reduction of linezolid use and infection control measures have been effective in controlling the dissemination of clinical isolates (21, 34). However, this genetic investigation indicates that pSCFS3-like plasmids are widely disseminated and can be transferred within staphylococci, warranting continued surveillance for resistance dissemination. In addition, *S. epidermidis* may act as a possible reservoir for resistance genes, since this species is still often considered a contaminant in clinical settings and determinants may spread unnoticed.

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