The Genetic Environment of the *cfr* Gene and the Presence of Other Mechanisms Account for the Very High Linezolid Resistance of *Staphylococcus epidermidis* Isolate 426-3147L

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The clinical *Staphylococcus epidermidis* isolate 426-3147L exhibits an unusually high resistance to linezolid that exceeds 256 μg/ml. The presence of the *cfr* gene, encoding the RNA methyltransferase targeting an rRNA nucleotide located in the linezolid binding site, accounts for a significant fraction of resistance. The association of *cfr* with a multicopy plasmid is one of the factors that contribute to its elevated expression. Mapping of the *cfr* transcription start sites identified the native *cfr* promoter. Furthermore, analysis of the *cfr* transcripts in *Staphylococcus epidermidis* 426-3147L showed that some of them originate from the upstream plasmid-derived promoters whose activity contributes to efficient *cfr* transcription. The genetic environment of the *cfr* gene and its idiosyncratic transcription pattern result in increased activity of Cfr methyltransferase, leading to a high fraction of the ribosomes being methylated at A2503 of the 23S rRNA. Curing of the *Staphylococcus epidermidis* 426-3147L isolate from the *cfr*-containing plasmid reduced the linezolid MIC to 64 μg/ml, indicating that other determinants contribute to resistance. Nucleotide sequence analysis revealed the presence of the C2534T mutation in two of the six 23S rRNA gene alleles as well as the presence of mutations in the genes of ribosomal proteins L3 and L4, which were previously implicated in linezolid resistance. Thus, the combination of resistance mechanisms operating through alteration of the drug target site appears to cause an unusually high level of linezolid resistance in the isolate.

The oxazolidinone antibiotic linezolid was introduced into clinical practice in 2000. Linezolid is active against most Gram-positive bacterial pathogens, including methicillin-resistant *Staphylococcus aureus* (MRSA), enterococci, and pneumococci (1). Linezolid targets the bacterial ribosome, where it binds in the catalytic peptidyl-transferase center (PTC) and inhibits protein synthesis (2–5). Due to the synthetic nature of the drug, it was anticipated that resistance to linezolid would be slow to develop. Indeed, during the first years after the introduction of linezolid into the market, resistant isolates appeared only sporadically, and all of the reported resistance mechanisms were confined to spontaneous ribosomal mutations. Mutations in rRNA at or near the drug binding site confer various levels of resistance depending on the type of mutation and the number of mutated *rrn* alleles. For example, the linezolid MIC of *Enterococcus faecium* may vary from 8 to 64 μg/ml depending on the number of mutated alleles (6). The most frequently found linezolid resistance mutation is the G2576T transversion (*Escherichia coli* numbering is used here and throughout) (7, 8). Resistance mutations have also been described for several other 23S rRNA positions (e.g., T2500A, G2447T, C2534T, G2215A, and T2504A) (reviewed in references 9 and 10). Aside from rRNA mutations, mutations in ribosomal proteins L4 and L3 have been found in some of the resistant isolates. While some of these mutations (e.g., a 6-bp deletion in the L4 gene) may play a direct role in linezolid resistance (11), the alterations in the L3 protein are usually found in combination with the 23S rRNA mutations and thus may help to decrease the fitness cost associated with changes in the rRNA structure (12–15).

The synthetic drug linezolid acts upon the ribosomal PTC, which is targeted by many natural antibiotics. Not surprisingly, natural resistance mechanisms that affect the PTC could render cells insensitive not only to natural antibiotics but also to linezolid. The *cfr* gene, whose product, the Cfr methyltransferase, modifies a 23S rRNA residue in the PTC, represents the first of such examples (16, 17). Cfr-directed C8 methylation of A2503 of 23S rRNA renders bacteria resistant to all of the peptidyl transferase-targeting antibiotics, including linezolid (18–20). The *cfr* gene was originally found in the plasmid pSCFS1 in a *Staphylococcus sciuri* strain isolated from cattle (16). In this strain, *cfr* is preceded by two overlapping open reading frames (ORFs), which code for peptides of 59 and 44 amino acids, respectively (21). While the role of these ORFs in controlling *cfr* expression has not been elucidated, their presence argues that *cfr* is inducible and, similar to several other resistance genes, could be controlled by translational attenuation (22, 23). Several other staphylococcal isolates from farm animals have been reported to harbor plasmids carrying *cfr*, where it is often associated with mobile genetic elements, such as transposons and insertion elements (24, 25).

In 2007, the first *cfr*-positive clinical isolate was reported (26). In this *S. aureus* isolate, designated CM05, the *cfr* gene was located in the chromosome, and its genetic environment differed greatly from that seen in the plasmids (19, 26, 27). In the CM05 isolate, *cfr* is located immediately downstream of the Tn917-derived gene *erm*(B), which encodes a methyltransferase that modifies A2058 in

23S rRNA and confers resistance to macrolides, lincosamides, and streptogramin B. Integration of *erm*(B) upstream of *cfr* resulted in the disruption of the putative *cfr* promoter and the deletion of the first 61 nucleotides (nt) of ORF1; it also placed *cfr* under the control of the *erm*(B) promoter. As a result, in the CM05 strain, *erm*(B) and *cfr* form an operon, *mlr*, whose expression confers resistance to all clinically relevant antibiotics that target the large ribosomal subunit (19, 26). Another Tn*917*-derived *erm*(B) gene is located downstream of the *cfr* gene. The 1.5-kb *cfr*-containing region is inserted in the 23S rRNA gene of the *rrn4* allele. Active transcription of the rRNA operon might contribute to *cfr* expression in the CM05 isolate (27).

Since 2007, *cfr* has been found in a number of linezolid-resistant clinical staphylococcal isolates, where it can be present on plasmids or in the chromosome (28–35). Association of *cfr* with mobile genetic elements and the low fitness cost associated with its expression (36) may account for its spread and maintenance in clinical pathogens.

Although *cfr* renders staphylococci linezolid resistant, the presence of the gene increases the MIC only severalfold, bringing it in the range of 8 to 32 μg/ml. Therefore, when the *cfr*-positive *S. epidermidis* strain 426-3147L, with a linezolid MIC exceeding 256 μg/ml, was isolated, the reasons for such a high level of resistance remained unclear (33). In this work, we examined the genetic environment of *cfr* in strain 426-3147L and characterized its expression. We also analyzed the presence of other genetic changes that can contribute to linezolid resistance of the 426-3147L isolate.

MATERIALS AND METHODS

Strains. *S. epidermidis* strain 426-3147L was isolated in 2007 through the LEADER Program from a blood culture of a 79-year-old female patient in Arizona (33) who had received vancomycin, cefepime, and ampicillin-sulbactam, although no linezolid use was documented. The same strain was isolated in the same facility 1 year later (30).

The *cfr*-positive *S. sciuri* strain carrying the plasmid pSCFS1 was isolated in 2000 from the nasal swab of a calf (16).

S. epidermidis ATCC 12228 is a linezolid-sensitive reference strain from the American Type Culture Collection.

DNA isolation. *S. epidermidis* and *S. aureus* cells were grown in brain heart infusion (BHI) media (BD Diagnostics, Sparks, MD). DNA was isolated using the MasterPure Gram-positive DNA purification kit (Epicentre Biotechnologies, Madison, WI) by following the manufacturer's protocol but with the inclusion of a 5-min bead-beating step after the addition of proteinase K (included in the kit).

RNA isolation. Total RNA was isolated from staphylococcal strains using the RNeasy Minikit (Qiagen, Germantown, MD) by following the manufacturer's protocol, with some modifications. Briefly, overnight cultures of cells grown in BHI media were diluted 1:100 in fresh media and grown to an A_{600} of 0.5. Five ml of cell culture was pelleted, washed with 500 μl H_2O, and resuspended in 200 μl of lysis buffer (Tris-HCl, pH 7.5, 30 mM $MgCl_2$, 30 mM NH_4Cl) containing 0.5 mg/ml of lysostaphin (Sigma, St. Louis, MO). After incubation for 30 min at 37°C, 350 μl of the kit buffer RLT was added, and the remaining steps of RNA isolation were performed as instructed by the kit manual.

Plasmid isolation. Total plasmid DNA was isolated from *S. epidermidis* or *S. aureus* using the PureYield Plasmid Midiprep system (Promega, Madison, WI), with minor modifications. Briefly, 50 ml of cells was pelleted and resuspended in 2.88 ml resuspension buffer (supplied in the kit) supplemented with 120 μg lysostaphin and 120 μg lysozyme (both from Sigma). The mixture was incubated at 37°C for 1 h, and the remaining steps were carried out by following the kit manual.

MICs. MICs were determined by broth microdilution according to the procedure established by the Clinical and Laboratory Standards Institute (37).

Detection of *cfr* by PCR. PCR for detection of the presence of *cfr* in staphylococci was carried out on whole cells. Colonies were picked from agar plates and incubated for 10 min in 10 μl of H_2O at 100°C. After 1 min of centrifugation, 1 μl of the resulting supernatant was used as a template in the PCR that was carried out with two *cfr*-specific primers, GAGATAACAGATCAAGTTTTA and CGAGTATATTCATTACCTCAT, under the following conditions: 2 min at 94°C; 30 cycles of 94°C for 30 s, 45°C for 30 s, and 72°C for 45 s; and a final extension at 72°C for 1 min.

Primer extension analysis of the A2503 modification. Primer extension analysis of the extent of A2503 methylation was carried out as previously described (17, 26), with minor modifications. Specifically, 1 pmol of [5′-^{32}P]-labeled DNA primer ACTGTCTCACGACGTTCT was annealed to 1.5 μg of *S. aureus* or *S. epidermidis* total RNA. The primer was extended with AMV reverse transcriptase (Seikagaku America, Falmouth, MA) in the presence of 1 mM each dGTP, dATP, and dCTP and 0.25 mM dTTP. The cDNA products were resolved in a denaturing 6% polyacrylamide sequencing gel.

Transformation of *S. aureus* RN4220 with the p7LC plasmid. Total plasmid DNA isolated from 426-3147L was transformed into the *S. aureus* strain RN4220 by electroporation (38). Transformants were selected on BHI agar pates containing 10 μg/ml florfenicol.

Mapping the *cfr* transcription start site. Twenty μg of total RNA isolated from *S. sciuri*, *S. aureus*, or *S. epidermidis* cells was annealed with 0.75 pmol of [5′-^{32}P]-labeled primer ATATCATTGTTTAGTTTAAAAAAGGACAG. The primer was extended with AMV reverse transcriptase (Seikagaku America) in 0.13 M Tris-HCl, pH 8.5, 10 mM $MgCl_2$, and 10 mM dithiothreitol (DTT) for 30 min at 40°C in the presence of 1 mM each dGTP, dATP, dCTP, and dTTP. The reactions were stopped by the addition of 1 μl of 10 N NaOH. After a 15-min incubation at 37°C, the reactions were neutralized with 1 μl of 10 N HCl. The cDNA products were ethanol precipitated and resolved in a denaturing 6% polyacrylamide sequencing gel alongside the sequencing reactions.

Curing 426-3147L of the p7LC plasmid. A culture of 426-3147L was grown overnight at 37°C in BHI media. The culture was streaked onto BHI agar plates and incubated at 42°C for 48 h. The colonies were restreaked onto fresh BHI plates and incubated for 24 h at 42°C. Once again, the colonies were restreaked onto a fresh BHI plate and maintained at 42°C for 48 h. Colonies from the final plate were replica plated on BHI agar plates without supplement or supplemented with 20 μg/ml florfenicol.

Sequencing *rrl*, *rplC*, and *rplD*. The individual 23S rRNA gene (*rrl*) alleles were PCR amplified using conditions described by Liakopoulos et al. (39) and sequenced. The genes *rplC* and *rplD*, encoding the ribosomal proteins L3 and L4, respectively, were PCR amplified according to Mendes et al. (34) and sequenced. Nucleotide and deduced amino acid sequences were compared to those from wild-type *S. epidermidis* ATCC 12228 (40).

Nucleotide sequence accession number. The sequence determined in the course of this work was deposited in GenBank under accession number JX910899.

RESULTS

Increased extent of A2503 modification in 426-3147L. The PCR analysis showed the presence of the *cfr* gene in the genome of 426-3147L (Fig. 1A), indicating that the activity of the Cfr methyltransferase accounts for at least a fraction of the linezolid resistance. Cfr-dependent methylation of C8 of A2503 in 23S rRNA interferes with the progression of reverse transcriptase (17), which makes it possible to use the primer extension technique for the evaluation of the extent of Cfr-mediated rRNA modification (19, 26). The appearance of a strong reverse transcriptase stop at A2503 in the RNA isolated from 426-3147L, which was lacking in
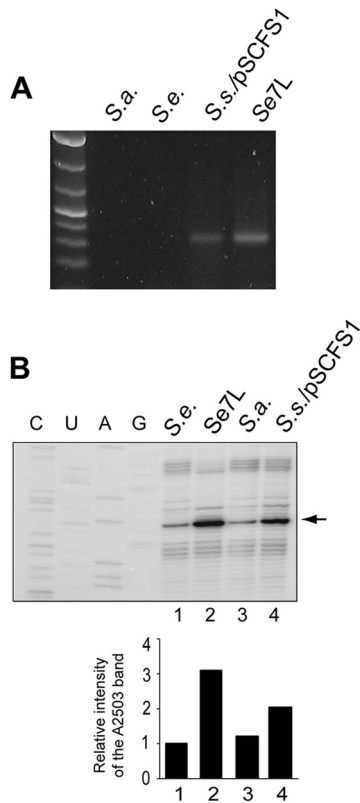
FIG 1 Linezolid resistance of strain 426-3147L correlates with expression of Cfr. (A) PCR analysis shows the presence of the *cfr* gene in 426-3147L. Linezolid-susceptible strains of *S. aureus* (RN4220) (*S.a.*) and of *S. epidermidis* (ATCC 12228) (*S.e.*) were used as negative controls. The *S. sciuri* strain carrying the pSCFS1 plasmid (*S.s.*/pSCFS1) was used as a positive control. (B) Primer extension analysis of the extent of Cfr-mediated C8 methylation of A2503 in the 426-3147L strain. Controls were the following: *S.e.*, *cfr*-negative *S. epidermidis* ATCC 12228; *S.a.*, *cfr*-negative *S. aureus*; *S.s.*/pSCFS1, *cfr*-positive *S. sciuri* carrying the pSCFS1 plasmid. The primer extension band corresponding to A2503 is indicated by an arrow. A weak reverse transcriptase stop in the *cfr*-negative control samples reflects C2 methylation of A2503 by an endogenous methyltransferase, RlmN (49). The bar graph below the gel shows the results of quantification of the band intensities.

control RNA samples, confirmed the efficient methylation of this nucleotide by the Cfr methyltransferase in 426-3147L (Fig. 1B). The extent of A2503 methylation in 426-3147L is ca. 1.5-fold higher than that in the *S. sciuri* strain carrying the pSCFS1 plasmid (Fig. 1B), indicating that the *cfr* gene structure, its copy number, or specific expression pattern could account for the elevated level of Cfr activity in the 426-3147L strain. It should be noted, however, that primer extension analysis allows only the relative quantification of A2503 modification, and it remains unknown which fraction of 23S rRNA molecules is modified in *S. sciuri*/pSCFS1 or 426-3147L.

The *cfr* gene is located on a plasmid in 426-3147L. The *cfr* gene is often found on plasmids (16, 24, 25, 41, 42). However, in some clinical and animal staphylococcal isolates it resides in the chromosome (25–27, 43). Our preliminary analysis showed that 426-3147L carries one or several plasmids that could host the *cfr* gene. In order to determine whether *cfr* was located on a plasmid or the chromosome, total DNA or plasmid preparation from 426-3147L was digested with the HindIII restriction enzyme and, after Southern blotting, hybridized with a *cfr*-specific probe (Fig. 2A and B). A probe specific for the chromosomal gene *se0011*, encoding homoserine-o-acetyltransferase, was included in the hybridization mixture as a control. While total DNA and the plasmid-enriched fraction contained DNA bands that hybridized with both probes (indicating contamination of the plasmid preparation with chromosomal material), the relative intensity of the *cfr*-specific signal was significantly stronger in the plasmid-enriched fraction, indicating that the *cfr* gene resides on the plasmid.

In order to unequivocally verify the presence of *cfr* on a plasmid, the plasmid preparation from 426-3147L was used to transform the *S. aureus* strain RN4220. Transformants were selected on plates containing 10 μg/ml florfenicol. The high florfenicol and linezolid MICs (Table 1) and PCR analysis (Fig. 2C) confirmed that *S. aureus* transformants acquired the *cfr* gene. HindIII restriction digest of the plasmid prepared from the transformed RN4220 cells (designated p7LC) generated five DNA fragments with a combined size of 30.5 kb, which provides the minimally estimated
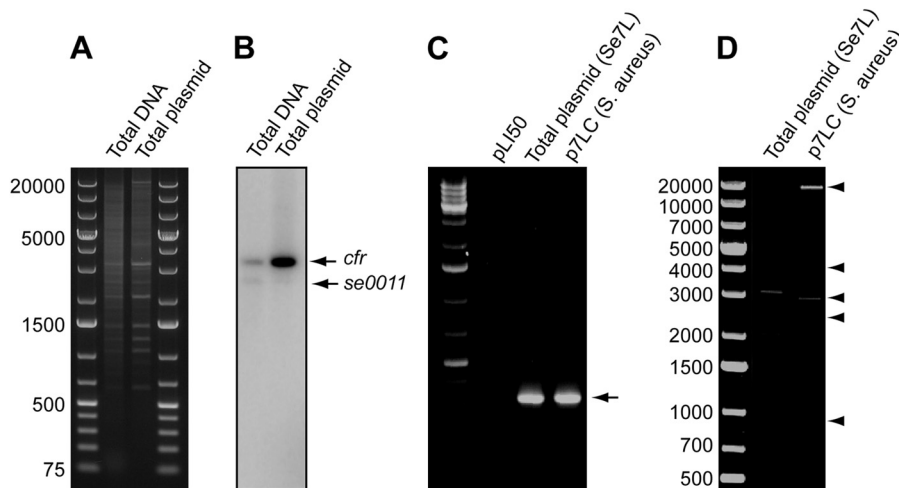
FIG 2 *cfr* gene in strain 426-3147L is located on a plasmid. (A) HindIII digest of total DNA or total plasmid preparation from 426-3147L. (B) The same gel after blotting and hybridization with the *cfr*-specific probe or probe for chromosomal gene *se0011*. (C) PCR detection of the *cfr* gene in the total plasmid preparation from 426-3147L or from *S. aureus* cells transformed with the plasmid prepared from 426-3147L. The plasmid vector pLI50 was used as a negative control. (D) HindIII digest of the total plasmid prepared from 426-3147L and from the transformed *S. aureus* cells. DNA bands attributed to the p7LC plasmid are indicated by arrowheads.

TABLE 1 MICs of linezolid and florfenicol for different strains used in the study

Organism and strain	MIC (µg/ml)	
	Linezolid	Florfenicol
S. epidermidis ATCC 12228	2	4
S. epidermidis 426-3147L	>256	1,024
S. aureus RN4220	2	4
S. aureus RN4220/p7LC	8	512
S. epidermidis 426-3147L/cured of p7LC	64	16

plasmid size. The p7LC HindIII fragments precisely matched a subset of HindIII fragments seen in the plasmid preparation from strain 426-3147L (Fig. 2D). The simplest explanation of this result is that the 426-3147L isolate carries two or more plasmids, one of which, p7LC, carries the *cfr* gene.

Although we did not make a special effort to accurately determine the copy number of the p7LC plasmid in the 426-3147L strain, the relative intensity of the p7LC-specific bands in the HindIII digest of the total DNA preparation (Fig. 2A) argues that the plasmid copy number can be in the range of 10 to 20 copies per chromosome, accounting for a high dosage of the *cfr* gene and, thus, likely contributing to the elevated level of Cfr expression.

Genetic organization of *cfr* in the p7LC plasmid. In order to determine the structure and genetic environment of the *cfr* gene in the p7LC plasmid, we sequenced a 10,125-bp segment of p7LC that included the 1,050-bp *cfr* gene, a 5,430-bp 5′-flanking region, and a 3,645-bp 3′-flanking region (accession no. JX910899) (Fig. 3). The nucleotide sequence of *cfr* is identical to that of the gene in the pSCFS1 plasmid and in the CM05 *S. aureus* clinical isolate (16, 26). The 367-bp upstream region of *cfr*, including the predicted *cfr* transcription start site (see below), closely matched the *cfr*-flanking sequence in pSCFS1, except for a 51-bp deletion and 2 single-nucleotide polymorphisms. The immediate genetic

environment of *cfr* in p7LC resembles that discovered in the recently described 40-kb plasmid pSS-01, which is found in staphylococcal isolates from swine (accession no. JQ041372) (25). A complete Tn*4001*-like transposon is found upstream of *cfr* in the p7LC plasmid. The aminoglycoside resistance gene *aacA-aphD* of Tn*4001* is flanked by two 1.3-kb IS*256* elements that carry transposase genes. As in pSS-01, the transposase genes in the left and right IS*256* elements in p7LC are not identical, although they do exhibit 87% sequence identity. Another copy of the IS*256*-associated transposase gene, oriented toward the *cfr* gene and identical in its sequence to the right (*cfr*-proximal) transposase gene of the Tn*4001*-like transposon, is found downstream of *cfr* (Fig. 3). Similar to pSS-01, this transposase gene is followed by a short ORF (ORF1) coding for a protein which exhibits homology to a transcription regulator of the *Paenibacillus vortex mer* operon (25, 44).

The target of insertion of the *cfr*-carrying genetic element is different in pSS-01 and p7LC (Fig. 3). In p7LC, the cassette is inserted in codon 84 of an ORF (ORF2) which exhibits 94% nucleotide sequence identity to the corresponding segment of a 398-codon ORF from the plasmid p18813-PO3, which is found in the *S. aureus* strain USA300 (45). The function of the protein encoded by ORF2 is unknown. The segment preceding ORF2 in p7LC, which includes another ORF (ORF3) coding for a hypothetical protein with unknown function, also shows high similarity (97% nucleotide identity) to the corresponding segment in p18813-PO3. The difference in the insertion loci of the *cfr*-carrying genetic element in pSS-01 and p7LC reveals its mobile nature and potential propensity for transposition between plasmids with various levels of species specificity.

Idiosyncratic transcription pattern of *cfr* in the p7LC plasmid. In the originally described *cfr*-bearing plasmid pSCFS1 (*S. sciuri*), expression of the *cfr* gene was proposed to be driven by a computationally predicted P*cfr* promoter located 366 bp upstream of the gene start codon (16). A similar hypothetical promoter sequence was found in the *cfr*-carrying plasmid pSCFS3 in *Staph-*
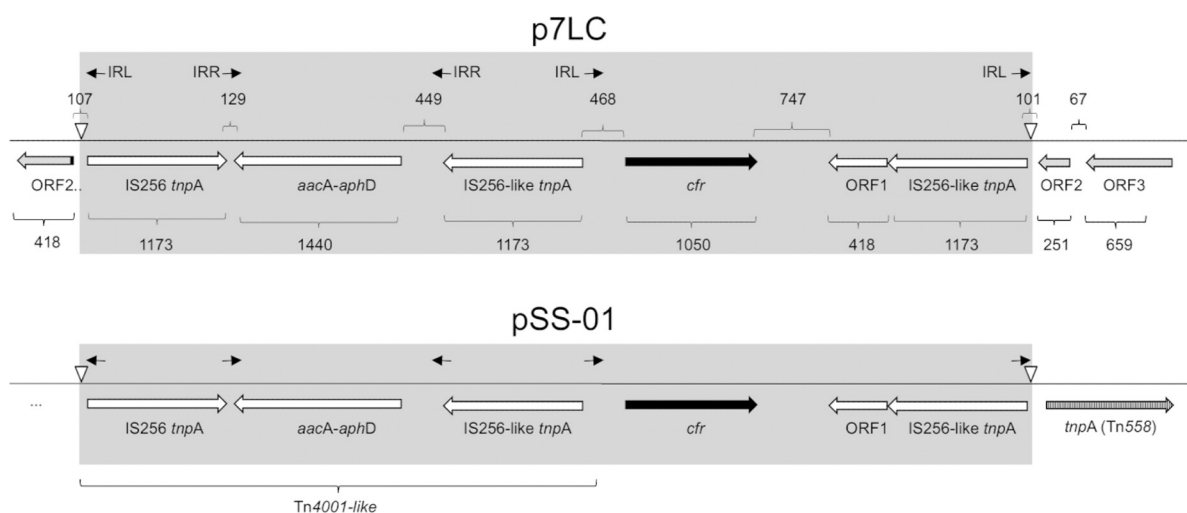
FIG 3 Genetic environment of *cfr* in p7LC. The *cfr* gene is shown in black. All of the other genes associated with the pSS-01-like element (gray) that are nearly identical between p7LC and pSS-01 are shown by open arrows. The genes at the insertion site of the element in p7LC are shown by diagonally hatched arrows. The Tn*558* transposase gene in the 3′-flanking region of the insertion site in pSS-01 is shown vertically hatched. The extended 5′-flanking sequence in pSS-01 is unknown; the immediate 5′-flanking sequence does not show homology to the site of insertion in p7LC. The left (IRL) and right (IRR) IS*256* inverted repeats are shown by arrows. The long inverted repeats at the site of the cassette insertion are indicated by open triangles. The gene sizes (in bp) are indicated below the genes; the sizes of intergenic regions are indicated above.

A

B
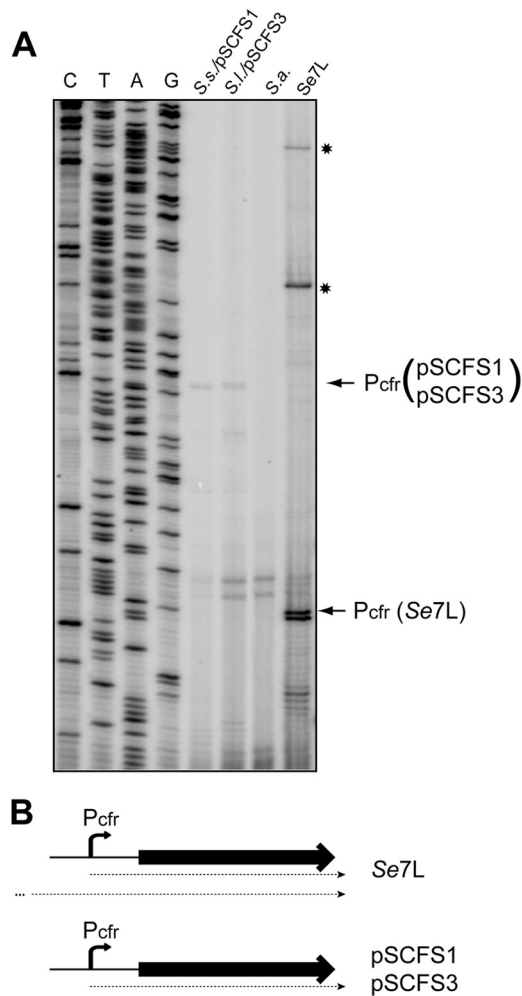
FIG 4 Analysis of the *cfr* transcription start sites. (A) Primer extension analysis was carried out on total RNA prepared from the *S. sciuri* strain carrying the pSCFS1 plasmid (*S.s.*/pSCFS1), *S. lentus* carrying pSCFS3 (*S.l.*/pSCFS3), *S. aureus* RN4220 (*S.a.*), or 426-3147L. The bands corresponding to cDNA products generated on transcripts initiated at P*cfr* are indicated by arrows. In the 426-3147L sample, this band is shifted because of a 51-bp deletion in the *cfr* leader region. The cDNA bands corresponding to the longer transcript(s) present in 426-3147L are indicated by asterisks. The pSCFS1 plasmid was used to generate the sequencing ladder. (B) Schematic representation of the *cfr* transcripts in 426-3147L and in staphylococcal strains carrying pSCFS1 or pSCFS3.

ylococcus lentus isolates (24). Since transcription from P*cfr* has not been experimentally verified, we wanted to test whether it indeed drives *cfr* expression and whether transcription from additional promoters could contribute to the expression of *cfr* in p7LC. We first tested if the hypothetical P*cfr* promoter directs the transcription of *cfr* in the pSCFS1 plasmid. Primer extension carried out on total RNA prepared from *S. sciuri* cells carrying the pSCFS1 plasmid showed an indicative band corresponding to the longest cDNA product, placing the transcription start at position 5924 of the reported sequence (accession no. NC_005076) (Fig. 4), thereby confirming that the originally predicted P*cfr* promoter indeed accounts for *cfr* expression in pSCFS1. We then proceeded with analyzing the *cfr* transcription pattern in the 426-3147L strain. While the band corresponding to transcripts initiated at P*cfr* were clearly detected, several longer primer extension products

were also observed (marked by asterisks in Fig. 4), suggesting that some of the transcripts which encompass the *cfr* gene in 426-3147L originated upstream of the P*cfr* transcription start site.

The sizes of the additional transcription products suggest that they were initiated within the Tn*4001*-like sequence whose right boundary is located 127 bp upstream from the *cfr* promoter start site. The right-most *tnp* gene of Tn*4001* (proximal to *cfr*) and the aminoglycoside resistance gene *aacA-aphD* of Tn*4001* are oriented in the direction opposite that of *cfr* (Fig. 3), so that promoters responsible for their transcription cannot contribute to *cfr* expression. Therefore, either a cryptic right-wise-oriented promoter within the *cfr*-proximal part of the Tn*4001*-like transposon or transcription from the promoter that drives expression of the left-most *tnp* gene of Tn*4001* accounts for additional transcripts that carry the *cfr* sequence. Irrespective of the exact location of the additional transcriptional start site(s), the presence of an additional promoter(s) that contributes to the transcription of *cfr* in the p7LC plasmid in the 426-3147L strain (and possibly in pSS-01) could lead to an elevated level of *cfr* mRNA and more efficient expression of the methylase than that of the strains where *cfr* is expressed exclusively from the P*cfr* promoter.

Determinants other than *cfr* contribute to the high level of linezolid resistance in the 426-3147L strain. Our analysis of the *cfr* genetic organization in the 426-3147L strain revealed that association of the gene with a multicopy p7LC plasmid and transcription from additional promoters could enhance its expression. However, none of these factors seem to be significant enough to account for the very high linezolid MIC of the 426-3147L strain (>256 μg/ml). Indeed, the linezolid MIC of *S. aureus* RN4220 transformed with p7LC reached only 8 μg/ml. Although this difference could be attributed to the genetic background of the host or to a different copy number of p7LC in *S. epidermidis* and *S. aureus*, it was likely that other resistance determinants were operating in the 426-3147L isolate. To verify this possibility, the 426-3147L cells were cured of the *cfr*-carrying p7LC plasmid. The loss of p7LC was confirmed by *cfr*-specific PCR. The cured strain exhibited a linezolid MIC of 64 μg/ml, which was notably reduced compared to the original, *cfr*-positive cells but substantially higher than the MIC of the linezolid-susceptible *S. epidermidis* control strain ATCC 12228 (Table 1). This result clearly showed that additional resistance mechanisms played a role in linezolid resistance of 426-3147L.

Besides the presence of the *cfr* gene, linezolid resistance can be conferred by mutations in 23S rRNA as well as in ribosomal proteins L3 and L4 (reviewed in references 9 and 10). To test for the presence of the resistance mutations, we sequenced domain V of each of the six individual *rrl* alleles of the 23S rRNA gene in 426-3147L as well as the genes *rplC* and *rplD*, encoding ribosomal proteins L3 and L4. The C2534T mutation (*E. coli* numbering) was identified in two of the six *rrl* alleles. This mutation was previously found in linezolid-resistant isolates of *S. epidermidis* (15, 46). Three missense mutations, altering amino acid residues His146Gln, Val154Leu, and Ala157Arg, were identified in the *rplC* gene, and the gene *rplD* had an insertion of a glycine codon (GGT) between the Gly71 codon and Arg72 codon. Although the direct contribution of the 23S rRNA mutation C2534T or the identified ribosomal protein mutations in linezolid resistance remains to be elucidated, their occurrence in the genes previously implicated in resistance to the drug and the general proximity to the site of

linezolid binding in the ribosome is highly suggestive of their role in increasing the linezolid MIC of the 426-3147L isolate.

DISCUSSION

In this paper, we have described a combination of mechanisms that account for the exceptionally high resistance of the clinical 426-3147L strain to linezolid. Of note, the finding of identical isolates of 426-3147L in the same health care facility in subsequent years stresses the ability of this strain to persist in a clinical setting, suggesting low combined fitness cost of the resistance mechanisms in 426-3147L and highlighting the importance of understanding the nature of linezolid resistance (30, 47).

One of the key factors of linezolid resistance of the 426-3147L strain is the presence of the *cfr* gene, which encodes the methyltransferase targeting an rRNA residue located in the linezolid binding site. The presence of the *cfr* gene on a multicopy plasmid, p7LC, increases the gene dosage to ca. 20 copies per cell. The close physical association of the *cfr* gene in p7LC with the Tn*4001*-like transposon apparently increases the rate of its transcription, because the *cfr*-specific transcripts originate not only at the authentic P$_{cfr}$ promoter but also within the transposon sequence (Fig. 4). The combination of these factors likely accounts for the enhanced expression of Cfr methyltransferase in the 426-3147L strain, a high degree of Cfr-mediated modification of A2503, and the resulting high level of linezolid resistance (Fig. 1B and Table 1).

In the first *cfr*-positive clinical MRSA isolate, *cfr* was genetically and transcriptionally linked to the *erm*(B) gene. Coexpression of the Cfr and Erm methyltransferases within the *mlr* operon rendered cells resistant to all of the clinically relevant antibiotics targeting the large ribosomal subunit, including drugs belonging to the macrolide, lincosamide, pleuromutilin, phenicol, oxazolidinone, and streptogramin families of compounds (19, 26). In 426-3147L, the presence of the Tn*4001*-like transposon upstream of *cfr*, which carries the aminoglycoside resistance gene *aacA-aphD*, links together resistance genes acting upon both ribosomal subunits. Given that the same Tn*4001-cfr* element is found on different plasmids within different genetic environments (Fig. 3), it is reasonable to expect dissemination of this element to other hosts and genetic locales.

The *cfr* gene is not the sole determinant of linezolid resistance in 426-3147L. Curing the strain of the *cfr*-containing p7LC plasmid reduced resistance from >256 to 64 μg/ml, indicating that other mechanisms contribute to the very high linezolid MIC of the original isolate. We found the C2534T mutation in two alleles of rRNA genes in the 426-3147L strain, as well as several mutations in proteins L3 and L4. The C2534T mutation has been found previously in *S. epidermidis* where, when it is present in two alleles, as is the case for the 426-3147L isolate, it increased the linezolid MIC to 8 μg/ml (48). The mutations found in 426-3147L in genes of proteins L3 and L4 are located close to the position where mutations were identified in other linezolid-resistant staphylococcal isolates (11–14, 28). These mutations can either increase the level of *cfr*-conferred linezolid resistance, as was previously noted for the ribosomal protein L3 mutations (28), or reduce the fitness cost associated with rRNA mutations or *cfr*-mediated rRNA modification.

The high level of linezolid resistance of the 426-3147L isolate seems to be accounted for by a combination of resistance mechanisms operating through the alteration of the drug target. The combined action of the *cfr*-mediated modification of an rRNA residue and mutations in rRNA and ribosomal proteins can be sufficient to provide the level of resistance observed in the isolate. Nevertheless, we cannot rule out the involvement of other resistance mechanisms, such as drug efflux or drug modification. Altogether, our findings reinforce a growing understanding that in spite of the synthetic nature of linezolid, the pathogens targeted by this antibiotic have sufficient genetic flexibility to acquire very significant levels of resistance by combining the effects of several different resistance mechanisms.

ACKNOWLEDGMENTS

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