

Identification and Characterization of the Multidrug Resistance Gene *cfr* in a Panton-Valentine Leukocidin-Positive Sequence Type 8 Methicillin-Resistant *Staphylococcus aureus* IVa (USA300) Isolate[∇]

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Received 11 August 2010/Returned for modification 24 September 2010/Accepted 27 September 2010

The staphylococcal *cfr* gene mediates resistance to phenicols, lincosamides, oxazolidinones, pleuromutilins, and streptogramin A, a phenotype that has been termed PhLOPS_A. The *cfr* gene has mainly been associated with coagulase-negative staphylococcal isolates from animals, and only a few *cfr*-positive methicillin-resistant *Staphylococcus aureus* (MRSA) isolates have been described so far. This study reports the first description of a *cfr*-positive MRSA isolate (M05/0060) belonging to the pandemic Panton-Valentine leukocidin (PVL)-positive sequence type 8 MRSA IVa/USA300 (ST8-MRSA-IVa/USA300) clone. The *cfr* gene was detected in M05/0060 using a DNA microarray which was used to screen PVL-positive MRSA isolates for the presence of virulence genes, typing markers, and antimicrobial resistance genes. Antimicrobial susceptibility testing revealed that M05/0060 exhibited the *cfr*-associated resistance phenotype. Molecular analysis identified the presence of *cfr* and a second phenicol resistance gene, *fexA*, on a novel 45-kb conjugative plasmid, which was designated pSCFS7. Within pSCFS7, a DNA segment consisting of *cfr*, a truncated copy of insertion sequence IS21-558, and a region with homology to the DNA invertase gene *bin3* of transposon Tn552 from *Bacillus mycoides* was integrated into the transposase gene *tnpB* of the *fexA*-carrying transposon Tn558. The emergence of a multidrug-resistant *cfr*-positive variant of ST8-MRSA-IVa/USA300 is alarming and requires ongoing surveillance. Moreover, the identification of a novel conjugative plasmid carrying the *cfr* gene indicates the ability of *cfr* to spread to other MRSA strains.

Methicillin-resistant *Staphylococcus aureus* (MRSA) is an important human pathogen and a significant cause of hospital-acquired infection and, more recently, community-acquired infection worldwide. Globally, different MRSA strains predominate in different geographical locations. In Ireland, a diverse range of MRSA strains have prevailed at different time periods (40), but for the last decade sequence type 22 MRSA IV (ST22-MRSA-IV) isolates have dominated in Irish hospitals (41), while ST30-MRSA-IVc and ST8-MRSA-IVa have predominated among community-acquired MRSA (CA-MRSA) isolates (37). Since ST8-MRSA-IVa, also known as USA300, was first reported in 2000 as a cause of skin and soft tissue infections in the United States, it has become the prevalent CA-MRSA strain in the United States, and evidence suggests that it is also emerging as a major nosocomial MRSA strain in the United States (6, 43). Originally, ST8-MRSA-IVa isolates harbored only the methicillin (*mecA*) and erythromycin [*msr(A)*] resistance genes, but they have since spread

worldwide while also acquiring additional resistance determinants, including *erm(A)* and *erm(C)*, *tet(M)* and *tet(K)*, and *mupA*, which encode resistance to macrolides-lincosamides-streptogramin B (MLS_B), tetracyclines, and mupirocin, respectively; most of these resistance determinants are located on plasmids (8, 12, 27, 43, 44). In addition, resistance to fluoroquinolones (levofloxacin), gentamicin, and trimethoprim-sulfamethoxazole and reduced susceptibility to vancomycin and daptomycin have also been observed among some ST8-MRSA-IVa/USA300 isolates (10, 11, 32, 43).

Mobile genetic elements, including plasmids, bacteriophages, pathogenicity islands, transposons, and chromosomal cassettes, play a vital role in the dissemination of virulence and antimicrobial resistance genes in *S. aureus* (3, 14, 24, 34–36, 47). The *cfr* gene can be plasmid or chromosomally located and encodes resistance to five classes of antimicrobial agents, i.e., phenicols, lincosamides, oxazolidinones, pleuromutilins, and streptogramin A, a phenotype that has been termed PhLOPS_A (23). Each of these antimicrobial classes contains antimicrobial agents that are used in the treatment of staphylococcal infections in either human or veterinary medicine, including chloramphenicol, florfenicol, clindamycin, lincomycin, pirlimycin, linezolid, retapamulin, tiamulin, valnemulin, and quinupristin-dalfopristin (23). The gene *cfr* encodes an rRNA methyltransferase that methylates the adenosine at position 2503 in 23S

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∇ Published ahead of print on 4 October 2010.

TABLE 1. Novel PCR primers used in the study

Gene or region amplified	Primer	Nucleotide sequence (5' → 3')	Nucleotide coordinates
<i>cfr</i>	cfrF1	GACTTTCGGCACCGGTAAT	3419–3437 ^a
	cfrR3	CAGTAGTCCATTCATATTTG	4681–4662 ^a
<i>fexA</i>	fexAF2	GCGTGATTCCCTAAGCTATT	7146–7164 ^a
	fexAR1	GAGAACCGAATCTTTAATCA	8800–8781 ^a
<i>radC</i>	radC-fw01	AGAGAAAATGCCAATGCAATCA	1736892–173687 ^b
	radC-rv02	TCACCGATTATAATATGATCCAATAAATCT	1736773–1736744 ^b

^a Nucleotide coordinates based on the nucleotide sequence of plasmid pSCFS3 (GenBank accession number AM086211).

^b Nucleotide coordinates based on the nucleotide sequence of MRSA strain COL (GenBank accession number CP000046.1).

rRNA (*Escherichia coli* 23S rRNA gene numbering) within the overlapping binding sites for these antimicrobial agents (20).

The *cfr* gene was originally identified in coagulase-negative staphylococci (CoNS) from animals (18, 39) but more recently has also been found in a very limited number of *Staphylococcus aureus* isolates and CoNS from humans (25, 26, 31, 45) and animals (16, 18). It is usually plasmid borne, and three plasmids, pSCFS1, pSCFS3, and pSCFS6 (15, 17, 18), have been characterized in detail. Plasmid pSCFS1 (17.1 kb) also harbors additional antimicrobial resistance genes, including *erm*(33), encoding MLS_B resistance, or *lsa*(B), encoding reduced susceptibility to lincosamides (17). The larger plasmids pSCFS3 (36 kb) and pSCFS6 (43 kb) also carry the Tn558-associated phenicol resistance gene *fexA*. In pSCFS3 and pSCFS6, *cfr* is integrated at different positions within the transposase genes *tnpA-tnpB* or *tnpC* of Tn558 (15, 18). In some staphylococcal isolates, *cfr* is located in the chromosomal DNA (15, 45). Recently, *cfr* has also been reported for the first time in a non-staphylococcal strain, *Bacillus* sp. strain BS-01 from swine feces in China (7). The *cfr* gene was detected on a novel 16.5-kb plasmid, pBS-01, in this *Bacillus* strain, along with transposon Tn917 harboring the MLS_B resistance gene *erm*(B) (7).

Here we report the first case of human clinical infection with an MRSA isolate belonging to the pandemic Pantone-Valentine leukocidin (PVL)-positive ST8-MRSA-IVa/USA300 strain harboring the multidrug resistance gene *cfr*. The aim of the present study was to comprehensively characterize the *cfr*-positive MRSA isolate and the genetic environment of the *cfr* gene.

MATERIALS AND METHODS

Bacterial isolates. A previously described collection (37) of Pantone-Valentine leukocidin (PVL)-positive MRSA isolates, recovered in Ireland between 1999 and 2005, was characterized using DNA microarrays (see below). One isolate (M05/0060) was found to harbor the multidrug resistance gene *cfr* and was characterized in detail in the present study. This isolate was originally recovered from a 26-year-old Irish male with a superficial scalp abscess (37).

Two ST8-MRSA-IVa/USA300 reference strains, for which full genome sequences are available (USA300-FPR3757 [GenBank accession no. CP000255] and USA300-TCH1516 [GenBank accession no. CP000730]), were included for comparison of DNA microarray hybridization profiles. The plasmid-free novobiocin-resistant *S. aureus* strain XU21 was used as a plasmid recipient in filter mating experiments (48).

Antimicrobial susceptibility testing. All PVL-positive MRSA isolates previously underwent antibiogram-resistogram (AR) typing using disk diffusion (37). Comparative antimicrobial susceptibility testing of M05/0060, its plasmid-cured derivative M05/0060-C1, the plasmid-free *S. aureus* recipient strain XU21, and its transconjugant derivative XU21-T1 (see below) was performed by broth microdilution as described in CLSI documents M07-A8 (4) and M100-S20 (5). In addition, further susceptibility tests on M05/0060 and the transconjugant derivative

XU21-T1 were performed using Vitek-2 (AST P580 panel, susceptibility tests for Gram-positive bacteria; bioMérieux, Nürtingen, Germany) and Etest (chloramphenicol, clindamycin, linezolid, and quinopristin-dalfopristin; bioMérieux) according to the manufacturer's protocols.

DNA microarray analysis. The StaphyType kit (Alere Technologies, Jena, Germany) was used for DNA microarray analysis of isolates, reference strains, and derivatives. The StaphyType kit consists of individual DNA microarrays mounted in eight-well microtiter strips which detect 334 *S. aureus* gene sequences, including species-specific, antimicrobial resistance, and virulence-associated genes, as well as typing markers (29, 30). Genomic DNA for use with the DNA microarray was extracted from all isolates by enzymatic lysis and using the Qiagen DNeasy kit (Qiagen, Crawley, West Sussex, United Kingdom) as described previously (29). The DNA microarray procedures were performed according to the manufacturer's instructions and have been described previously in detail (28, 29).

Molecular typing. Pulsed-field gel electrophoresis (PFGE); *spa*, staphylococcal cassette chromosome *mec* (SCC*mec*), and toxin gene typing; and multilocus sequence typing (MLST) analysis of the PVL-positive MRSA isolates were performed as described previously (37, 42). The *cfr*-positive MRSA isolate M05/0060 also underwent direct repeat unit (*dru*) typing, which was performed as described previously (41).

Plasmid analysis. Plasmid curing was performed by culturing M05/0060 in brain heart infusion (BHI) (Oxoid Ltd., Hampshire, United Kingdom) broth at 43°C for 24 h at 200 rpm. This was followed by subculture into fresh BHI broth and incubation as before for five consecutive rounds, after which individual colonies obtained following plating on BHI agar were screened for loss of resistance to chloramphenicol (30 µg/ml) by replica plating. Conjugative transfer of *cfr* to the plasmid-free, novobiocin-resistant *S. aureus* recipient strain XU21 was conducted by filter mating (49). Putative transconjugants were plated onto BHI agar containing 100 µg/ml novobiocin to select for the recipient strain and 30 µg/ml chloramphenicol to select for a putative *cfr*-carrying plasmid. Putative cured derivatives and transconjugants underwent DNA microarray analysis and comparative antimicrobial susceptibility testing as described above. Plasmid DNAs from M05/0060, its plasmid-cured derivative M05/0060-C1, and a transconjugant derivative of XU21 (XU21-T1) were extracted and purified using a modified alkaline lysis method (38). The size of the *cfr*-carrying plasmid detected in the transconjugant derivative XU21-T1 was determined based on the sum of the fragment sizes obtained following digestion of plasmid DNA with the restriction endonuclease BglII (Roche Diagnostics GmbH, Mannheim, Germany).

Molecular characterization of the novel *cfr*- and *fexA*-carrying plasmid pSCFS7. The *cfr* and *fexA* genes of M05/0060 were amplified and sequenced using primers cfrF1/cfrR3 and fexAF2/fexAR1, respectively (Table 1), using GoTaq DNA polymerase (Promega, Madison, Wisconsin) according to the manufacturer's instructions and the following conditions: 94°C for 2 min; 34 cycles of 95°C for 30 s, 50°C for 2 min, and 72°C for 1 min; and a final extension at 72°C for 10 min. PCR products were purified using the Genelute PCR cleanup kit (Sigma-Aldrich Chemical Co. Tallaght, Dublin, Ireland). DNA sequencing of amplifiers was performed commercially by Geneservice (Source Bioscience, St. James's Hospital, Dublin, Ireland).

To investigate whether *cfr* was associated with the *fexA*-carrying transposon Tn558 in the *cfr*-carrying plasmid pSCFS7 identified in M05/0060, plasmid DNAs from M05/0060 and the transconjugant derivative XU21-T1 harboring pSCFS7 were subjected to PCR analysis for the different regions of Tn558. The transposase genes *tnpA*, *tnpB*, and *tnpC* and the region from *tnpB-fexA* were amplified from M05/0060 and XU21-T1 using previously described primers and conditions (18). Since the *tnpB* amplicon obtained was larger than expected for Tn558 in

both strains, PCRs were also performed using the gel-purified *tnpB* amplification product obtained with template DNAs from both organisms using previously described *cfr*-specific primers (18). Gel purification was performed using the QiaexII PCR cleanup kit (Qiagen). The resulting amplicon from M05/0060 was cloned as described previously (18) and sequenced by primer walking (MWG, Ebersberg, Germany).

Primers radC-fw01 and radC-rv02 (Table 1) were designed and used to amplify the *radC* gene covering the potential chromosomally located *cfr* integration site in M05/0060, using *Taq* DNA polymerase and the following conditions: 95°C for 2 min; 35 cycles of 95°C for 30 s, 64°C for 20 s, and 72°C for 2 min; and a final extension at 72°C for 5 min.

Nucleotide sequence accession numbers. The nucleotide sequences of *cfr*, *fexA*, and the 4,043-bp *tnpB* amplicon of M05/0060 have been deposited in GenBank under accession numbers FN995111, FN995110, and FR675942, respectively.

RESULTS

Phenotypic and genotypic characteristics of M05/0060. M05/0060 exhibited the same MLST (ST8) and *spa* (t008), *dru* (dt9g), and SCCmec (IVa) types as the ST8-MRSA-IVa/USA300 clone (44). In the present study, DNA microarray analysis of M05/0060 and comparison with DNA microarray data for two whole-genome-sequenced ST8-MRSA-IVa/USA300 reference strains (FPR3757 and TCH1516) (9, 13), revealed that M05/0060 was indistinguishable from the ST8-MRSA-IVa/USA300 strain TCH1516, apart from carriage of *cfr* and *fexA* (Table 2). Antimicrobial susceptibility testing revealed that M05/0060 exhibited the *cfr*-associated resistance phenotype (PhLOPS_A), including resistance to chloramphenicol, florfenicol, clindamycin, linezolid, tiamulin, virginamycin M₁, and quinupristin-dalfopristin (Table 3).

Confirmation of the presence of *cfr* and *fexA*. The presence of *cfr* and *fexA* in M05/0060 was confirmed by PCR and sequencing of the entire *cfr* and *fexA* genes. The *cfr* sequence of M05/0060 exhibited 100% identity with *cfr* of pSCFS1 (GenBank accession number NC_005076.1) (17) and pSCFS6 (AM408573.1) (15) and 99.9% identity with *cfr* of pSCFS3 (AM086211.1) (18) due to a single nucleotide difference at nucleotide position 761 of *cfr* with no resulting amino acid change. The *fexA* sequence of M05/0060 exhibited 100% identity with *fexA* of *Bacillus* sp. strain BS-01 (GU591496.1) and 99.9% identity with *fexA* of pSCFS3 (AM086211.1), *Staphylococcus lentus* (AJ549214.1 and AJ715531.1), *Staphylococcus warneri* (AM408573.1), and *Staphylococcus simulans* (AM086400.1) due to two nucleotide differences at nucleotide positions 391 and 913, which resulted in two amino acid changes, from isoleucine to valine and valine to isoleucine, respectively.

Localization of *cfr* and *fexA* in M05/0060 on the novel plasmid pSCFS7. The *radC* integration site in M05/0060 was examined using primers spanning this integration site, because to date *cfr* has been reported to be collocated with *tnpB* on transposon Tn558 and this transposon as well as other members of the Tn554 transposon family are known to integrate specifically into *radC* (19, 33). This can, for instance, be observed in the published genome sequence for *S. aureus* Mu50 (GenBank accession number BA000017) or in other *S. aureus* strains which carry Tn554 (22). As PCR using primers spanning this integration site yielded a product of the expected length (approximately 150 nucleotides), it was assumed that (i) *cfr* was not located there and (ii) it was not localized on a chromosomally integrated Tn554/Tn558-like transposon.

Dilution series of unfragmented and RNA-free M05/0060 total cellular DNA were subjected to DNA microarray analysis, and signals for *fexA* and *cfr* were significantly more intense in stepwise-diluted samples than all other species markers, including 23S rRNA. Since the microarray method uses a linear labeling and amplification procedure, the higher signal intensity observed for *fexA* and *cfr* relative to housekeeping genes suggested that M05/0060 harbored multiple, most likely plasmid-borne copies of these genes.

The plasmid location of *cfr* and *fexA* in M05/0060 was confirmed by curing and conjugation experiments. Plasmid profiling identified the presence of a ca. 45-kb plasmid in M05/0060, which was absent in its cured derivative M05/0060-C1 and present in the transconjugant XU21-T1. Comparative susceptibility testing revealed the presence of the *cfr*-associated resistance phenotype in XU21-T1 but its absence in the XU21 parental strain and the plasmid-cured derivative M05/0060-C1 (Table 3). Moreover, microarray analysis confirmed that M05/0060-C1 and XU21-T1 were indistinguishable from M05/0060 and XU21 except for the absence and the presence of *cfr* and *fexA*, respectively (Table 2). Following the purification of plasmid DNA from transconjugant XU21-T1, this ca. 45-kb conjugative plasmid was found to differ in its BglII restriction pattern from the previously described nonconjugative, *cfr*-carrying plasmids pSCFS1, pSCFS3, and pSCFS6 (15, 17, 18), and it was therefore designated novel plasmid pSCFS7.

Molecular characterization of the genetic environment of *cfr* on plasmid pSCFS7. When *cfr* and *fexA* have previously been identified on the same plasmid, the *cfr* gene and its flanking regions were found to be integrated into the transposase genes of the *fexA*-carrying transposon Tn558 (15, 18). Tn558-directed PCRs revealed that while the amplicons for *tnpA*, *tnpC*, and *tnpB-fexA* obtained using M05/0060 and transconjugant XU21-T1 template DNAs corresponded in size to those obtained for a complete Tn558, the *tnpB* amplicon obtained with both M05/0060 and XU21-T1 was approximately 2.5 kb larger than expected. PCR detection of *cfr* using this gel-purified larger *tnpB* amplicon obtained from both M05/0060 and the transconjugant XU21-T1 as templates and primers cfr-fw and cfr-rv (18) yielded an amplicon of the expected size for both strains, which suggested that a *cfr*-carrying DNA segment had been integrated into the *tnpB* reading frame in pSCFS7. To confirm this hypothesis, the *tnpB* amplicon from M05/0060 was cloned and sequenced. The entire *tnpB* amplicon of pSCFS7 comprised 4,043 bp and consisted of initial (positions 1 to 859) and terminal (positions 3826 to 4043) *tnpB* segments (Fig. 1). A largely truncated IS21-558 element was detected (positions 846 to 1131), of which only the 3' terminus of *istBS* and the downstream region, including the terminal inverted repeat, were present. The region downstream of this IS21-558 relic (positions 1132 to 3254), including the entire *cfr* gene (positions 1622 to 2671), exhibited 99.9% nucleotide sequence identity to the corresponding *cfr* sequences of pSCFS3 and pSCFS6 (15, 18). The adjacent 187 bp did not reveal significant homology to sequences deposited in the databases but were followed by the 5' end of a reading frame (positions 3442 to 3825) whose 127-amino-acid product revealed 78% identity and 90% similarity to the transposon Tn552 DNA-invertase Bin3 from *Bacillus mycoides* Rock3-17 (ACMW01000216) (Fig. 1). Analysis of the boundaries of this integrated DNA segment revealed

TABLE 2. DNA microarray hybridization profiles of the two USA300 reference strains FPR3757 and TCH1516, M05/0060, its cured derivative M05/0060-C1, the *S. aureus* recipient strain XU21, and its transconjugant derivative XU21-T1^a

Class	Gene(s)	Hybridization result for ^b :					
		USA300-FPR3757	USA300-TCH1516	M05/0060	M05/0060-CI	XU21	XU21-T1
Species markers	<i>katA, coa, nuc, spa</i>	POS	POS	POS	POS	POS	POS
<i>agr</i> group	<i>agr</i> group I	POS	POS	POS	POS	POS	POS
SCCmec-associated markers	<i>mecA</i>	POS	POS	POS	POS	NEG	NEG
	<i>ΔmecR</i>	POS	POS	POS	POS	NEG	NEG
	<i>mecR/1</i>	NEG	NEG	NEG	NEG	NEG	NEG
	<i>ccrA/B-1</i>	NEG	NEG	NEG	NEG	NEG	NEG
	<i>Q9XB68-dcs</i>	POS	POS	POS	POS	NEG	NEG
	<i>ccrA/B-2</i>	POS	POS	POS	POS	NEG	NEG
	<i>kdp</i> -SCC locus	NEG	NEG	NEG	NEG	NEG	NEG
	<i>ccrA/B-3</i>	NEG	NEG	NEG	NEG	NEG	NEG
	<i>ccrC, ccrA/B-4</i>	NEG	NEG	NEG	NEG	NEG	NEG
Antimicrobial resistance genes	<i>blaZ/II/R</i>	NEG	POS	POS	POS	NEG	NEG
	<i>erm(A), erm(B)</i>	NEG	NEG	NEG	NEG	NEG	NEG
	<i>erm(C)</i>	POS	NEG	NEG	NEG	NEG	NEG
	<i>lhu(A)</i>	NEG	NEG	NEG	NEG	NEG	NEG
	<i>msr(A), mph(C)</i>	NEG	POS	POS	POS	NEG	NEG
	<i>aacA-aphD</i>	NEG	NEG	NEG	NEG	NEG	NEG
	<i>aadD</i>	NEG	NEG	NEG	NEG	NEG	NEG
	<i>aphA3/sat</i>	NEG	POS	POS	POS	NEG	NEG
	<i>dfrrS1</i>	NEG	NEG	NEG	NEG	NEG	NEG
	<i>far1</i>	NEG	NEG	NEG	NEG	NEG	NEG
	<i>mupA</i>	POS	NEG	NEG	NEG	NEG	NEG
	<i>tet(K)</i>	POS	NEG	NEG	NEG	NEG	NEG
	<i>tet(M)</i>	NEG	NEG	NEG	NEG	NEG	NEG
	<i>cat</i>	NEG	NEG	NEG	NEG	NEG	NEG
	<i>cfr</i>	NEG	NEG	POS	NEG	NEG	POS
<i>fexA</i>	NEG	NEG	POS	NEG	NEG	POS	
Virulence-associated genes	<i>tstI</i>	NEG	NEG	NEG	NEG	NEG	NEG
	<i>sea, seb, see, seh</i>	NEG	NEG	NEG	NEG	NEG	NEG
	<i>sec/l, sed/j/r</i>	NEG	NEG	NEG	NEG	NEG	NEG
	<i>seg/i/m/n/o/u</i>	NEG	NEG	NEG	NEG	NEG	NEG
	<i>sek/q</i>	POS	POS	POS	POS	NEG	NEG
	<i>lukF/S-PV</i>	POS	POS	POS	POS	NEG	NEG
	<i>lukF/S-hlg, hlgA</i>	POS	POS	POS	POS	POS	POS
	<i>sak/chp/scn</i>	POS	POS	POS	POS	NEG	NEG
	<i>etA/B/C</i>	NEG	NEG	NEG	NEG	NEG	NEG
	<i>edinA/B/C</i>	NEG	NEG	NEG	NEG	NEG	NEG
	<i>arcA/B/C/D</i>	POS	POS	POS	POS	NEG	NEG
Capsule type	Capsule type 5	POS	POS	POS	POS	POS	POS
	Capsule type 8	NEG	NEG	NEG	NEG	NEG	NEG

^a The StaphyType kit (Alere Technologies, Jena, Germany) was used for DNA microarray analysis of isolates, reference strains, and derivatives. Full data sets are available upon request.

^b POS, positive (i.e., yielded a hybridization signal corresponding to the gene indicated); NEG, negative (i.e., failed to yield a hybridization signal corresponding to the gene indicated). FPR3757 and TCH1516 are two USA300 reference strains for which full-genome sequences are available (accession numbers CP000255 and CP000730, respectively).

two potential recombination sites, one of 14 bp between *tnpB* and *istBS* and another of 13 bp between *bin3* and *tnpB* (Fig. 2). These putative recombination sites are assumed to play a role in the integration of this 2,980-bp segment and the concomitant 433-bp deletion of *tnpB*.

DISCUSSION

This is, to our best knowledge, the first report of a human infection with the major pandemic MRSA clone ST8-MRSA-IVa/USA300 harboring the multidrug resistance gene *cfr*. Iso-

lates of this PVL-positive MRSA clone are commonly associated with skin and soft tissue infections but also with life-threatening necrotizing pneumonia. Therapy options for the latter condition are very limited, since glycopeptide antibiotics tend to have poor penetration into lung tissue and reduced susceptibility to vancomycin has been observed among MRSA isolates, including USA300 (10, 11). Daptomycin cannot be used in cases of pneumonia, as it reacts with lung surfactant (2). While rifampin, levofloxacin, and fosfomycin are viable options, resistance to these antimicrobial agents is common and can emerge during therapy. The synergistic effect of sul-

TABLE 3. Antimicrobial resistance profiles of M05/0060 harboring the *cfr*-encoding conjugative plasmid pSCFS7, its cured derivative M05/0060-C1 lacking pSCFS7, *S. aureus* recipient strain XU21, and its transconjugant derivative XU21-T1 harboring pSCFS7

Isolate or derivative	<i>cfr</i> and <i>fexA</i> carriage	MIC ($\mu\text{g/ml}$) ^a									
		TIA	VIR M ₁	Q-D	LZD	CLI	CHL	FFC	ERY	OXA	VAN
MRSA M05/0060	<i>cfr</i> , <i>fexA</i>	≥ 128	32	2	8	≥ 128	256	256	≥ 64	≥ 32	1
MRSA M05/0060-C1		1	1	0.5	1	0.25	8	4	≥ 64	≥ 32	1
<i>S. aureus</i> XU21		1	1	0.25	1	0.25	8	4	0.5	0.12	1
<i>S. aureus</i> XU21-T1	<i>cfr</i> , <i>fexA</i>	≥ 128	32	2	4	≥ 128	128	256	0.5	0.12	1

^a TIA, tiamulin; VIR M₁, virginamycin M₁; Q-D, quinupristin-dalfopristin; LZD, linezolid; CLI, clindamycin; CHL, chloramphenicol; FFC, florfenicol; ERY, erythromycin; OXA, oxacillin; VAN, vancomycin. Resistance to tiamulin, virginamycin M₁, quinupristin-dalfopristin, linezolid, clindamycin, chloramphenicol, and florfenicol is indicative of the PhLOPS_A phenotype.

fonamide and trimethoprim under *in vivo* conditions is in dispute, which limits its use in life-threatening conditions. Ceftobiprole is not yet available in most countries, and for tigecycline, clinical experience is still lacking. Thus, treatment of necrotizing pneumonia and of other serious infections caused by PVL-positive MRSA relies primarily on vancomycin and oxazolidinones, such as linezolid, and resistance to the latter is encoded by *cfr* or can be due to mutations in the domain V region in the 23S rRNA gene (46).

The *cfr* gene identified in the ST8-MRSA-IVa isolate M05/0060 in the present study was located, along with *fexA*, on a novel 45-kb conjugative plasmid designated pSCFS7. There have been two previous reports of plasmids that carry both *cfr* and *fexA* in staphylococci, including pSCFS3 and pSCFS6. Both of these plasmids have been detected only in staphylococcal isolates from animals (15, 16, 18), and in each plasmid *cfr* is integrated into the *fexA*-carrying transposon Tn558 (15, 18). In pSCFS3 and pSCFS6, the Tn558 elements are disrupted and in part truncated by the insertion of DNA segments of 4,674 bp and 9,594 bp, respectively, which comprise the *cfr*

gene along with one or two copies of the insertion sequence IS21-558 and, in the case of pSCFS6, the clindamycin resistance gene *lsa(B)* (15, 18). In contrast, in the novel plasmid pSCFS7, the *tnpB* gene of Tn558 was disrupted and in part deleted by the insertion via recombination of a DNA segment of 2,980 bp carrying *cfr*, a truncated IS21-558 (upstream of *cfr*), and $\Delta bin3$ (downstream of *cfr*) (Fig. 1). The *bin3* gene encodes a DNA invertase and has previously been reported only in the transposon Tn552 from *Bacillus mycoides*. These observations, coupled with the recent report of *cfr* in a *Bacillus* sp. isolate (7), point toward the mobility of *cfr*, its ability to spread to different bacterial species, and the role that recombination processes play in the stable fixation of *cfr* in new vector plasmids.

The *cfr*-positive ST8-MRSA-IVa isolate M05/0060 harboring *cfr* on the novel plasmid pSCFS7 differs significantly from at least two of the three previously reported cases of *cfr*-positive *S. aureus* isolates recovered from humans and from those from animals. A *cfr*-positive MRSA isolate from a Colombian patient belonged to ST5/ST221-MRSA-I, and *cfr* was chromosomally located (1, 45). A *cfr*-positive methicillin-susceptible *S.*

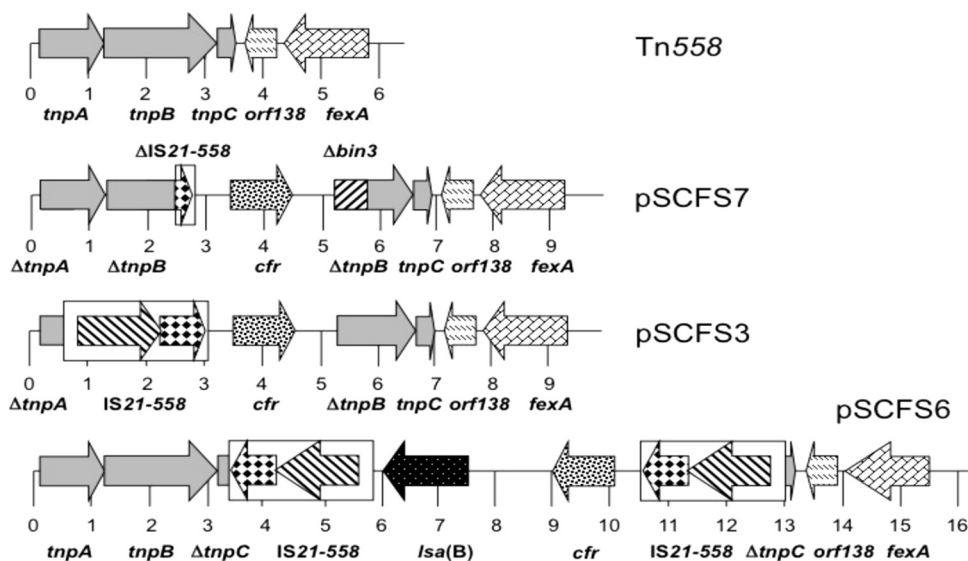


FIG. 1. Schematic diagram showing the organization of the novel variant of transposon Tn558 of plasmid pSCFS7 (accession no. FR675942) relative to Tn558 (AJ715531) and the Tn558 variants harbored by plasmids pSCFS3 (AM086211) and pSCFS6 (AM408573). The positions and orientations of the genes coding for transposition functions (*tnpA*, *tnpB*, and *tnpC*), antimicrobial resistance [*fexA*, resistance to florfenicol and chloramphenicol; *cfr*, resistance to phenicols, lincosamides, oxazolidinones, pleuromutilines, and streptogramin A antibiotics; *lsa(B)*, reduced susceptibility to lincosamides], or unknown functions (*orf138*) are indicated by arrows. Δ , truncated gene. A distance scale in kb is shown below each map.

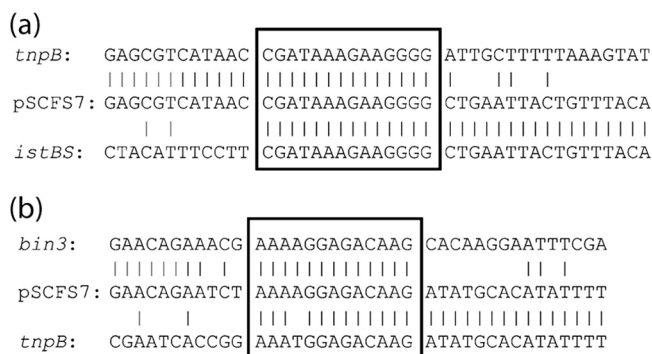


FIG. 2. Potential sites used for the integration of the 2,966-bp *cfr*-containing segment into the *tnpB* reading frame, thereby causing a 433-bp deletion of *tnpB*. (a) Recombination site between the 5' end of *tnpB* and IS21-558. Displayed are the *tnpB* sequence at positions 3369 to 3411 of plasmid pSCFS6 (accession no. AM408573), the *istBS* sequence at positions 4724 to 4682 of plasmid pSCFS6, and the sequence of pSCFS7 at positions 834 to 878. (b) Recombination site between *bin3* and the 3' end of *tnpB*. Displayed are the *bin3* sequence of *B. mycooides* at positions 4550 to 4512 (ACMW01000216), the *tnpB* sequence at positions 3726 to 3764 of plasmid pSCFS6, and the sequence of pSCFS7 at positions 3811 to 3853. Nucleotides identical to the sequence of pSCFS7 are indicated by vertical bars. The recombination sites, where crossover is believed to have occurred, are boxed.

aureus (MSSA) isolate from a patient in a hospital in the United States was a sporadic strain, and *cfr* was located on a 55-kb plasmid that was not further characterized (26). However, the *cfr*-positive MRSA isolates reported from an outbreak in a Spanish hospital were not investigated for the location of *cfr* (31), and while these isolates appeared to be closely related by PFGE, no details of MLST or SCCmec types were provided, so comparison to international MRSA strains is not possible (31). Reports of *cfr* among *S. aureus* isolates from animals have all involved pigs, including isolates belonging to ST9-MSSA and ST398-MRSA-V, and all these isolates harbored *cfr* on plasmid pSCFS3 (16, 18).

While *cfr* is still rare among *S. aureus* isolates, it appears to be more abundant among CoNS, and it is evident that CoNS from both humans and animals may act as a reservoir for *cfr* and other resistance genes in *S. aureus*. The *cfr* gene was first reported in 2000 from a bovine *Staphylococcus sciuri* isolate and has since been identified among additional *S. sciuri* isolates as well as in isolates of *S. warneri*, *S. simulans*, *S. hyicus* and *S. lentus*, all from animal sources (15, 18, 39). These CoNS from animals all harbored *cfr* on plasmid pSCFS1, pSCFS3, or pSCFS6 (15, 17, 18). In addition, there have been two recent reports of CoNS from humans harboring *cfr*, including two *Staphylococcus epidermidis* isolates from Mexico, in which the location of *cfr* was not determined (25), and the United States, in which *cfr* was localized on a 175-kb plasmid (26). While there have been no reports of *cfr*-positive staphylococci from Ireland prior to the present study, linezolid resistance has been reported among three clinical *S. epidermidis* isolates (21). However, it was not determined if *cfr* was present in these *S. epidermidis* isolates, and the linezolid resistance phenotype in these isolates may alternatively have been due to the presence of a mutation in the 23S rRNA gene. Interestingly, during the present study, problems in interpreting linezolid MICs using the Vitek-2 software were encountered. The Vitek-2 software

reported a linezolid MIC of 8 μ g/ml for the *cfr*-positive MRSA isolate M05/0060, which is above the resistance breakpoint for linezolid (Table 3). However, the Vitek-2 software recommended that the linezolid susceptibilities should be manually changed to 4 μ g/ml and that the isolate should be reported as susceptible to linezolid. While the authors of a 2008 study (1) did not encounter problems using the Vitek system, they did report problems with detecting *cfr*-mediated linezolid resistance using disk diffusion and Etest. The authors found that a *cfr*-positive MRSA isolate was reported as linezolid susceptible after 24 h of incubation with the Etest and suggested that a longer incubation period was required to detect this resistance phenotype (1).

Since ST8-MRSA-IVa/USA300 has proved to be a successful clone capable of epidemic spread, the emergence of a *cfr*-positive variant of this strain is cause for significant concern and warrants close surveillance. The localization of *cfr* on a novel conjugative plasmid and the identification of a potential novel integration site within Tn558 indicate that *cfr* has the ability to spread to other MRSA strains. Our finding of *cfr* in ST8-MRSA-IVa/USA300 would almost certainly not have occurred if we had not used the DNA microarray to screen for the presence of an extensive range of virulence and antimicrobial resistance genes among large numbers of MRSA isolates. Our findings highlight the effectiveness of such high-throughput systems for ongoing surveillance of MRSA.

ACKNOWLEDGMENTS

This work was supported by the Microbiology Research Unit, Dublin Dental School and Hospital, and the Friedrich-Loeffler Institut.

We thank Kerstin Meyer for excellent technical assistance with work undertaken at the Friedrich-Loeffler Institut. The *S. aureus* recipient strain XU21 and USA300 strains FPR3757 and TC1516 were kindly provided by Edet Udo, Kuwait University, Geoffrey Coombs, Royal Perth Hospital, Perth, Australia, and Sarah K. Highlander, Baylor College of Medicine, Houston, TX, respectively.

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