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Characterization of novel conjugative multiresistance plasmids carrying *cfr* from linezolid-resistant *Staphylococcus epidermidis* clinical isolates from Italy

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Objectives: The objective of this study was to investigate the genetic environment of the *cfr* gene from two linezolid-resistant clinical isolates of *Staphylococcus epidermidis* from Italy.

Methods: The two strains (SP1 and SP2) were phenotypically and genotypically characterized. Transferability of *cfr* was assessed by electrotransformation and conjugation. The genetic contexts of *cfr* were investigated by PCR mapping, sequencing and comparative sequence analyses.

Results: SP1 and SP2 belonged to ST23 and ST83, respectively. In both strains, the *cfr* gene was located on a plasmid, which could be transferred to *Staphylococcus aureus* by transformation and conjugation. In isolate SP1, linezolid resistance mediated by mutations in 23S rRNA and the L3 ribosomal protein was also detected. pSP01, the *cfr*-carrying plasmid from strain SP1, had a larger number of additional resistance genes and was sequenced (76 991 bp). It disclosed a distinctive mosaic structure, with four cargo regions interpolated into a backbone 95% identical to that of *S. aureus* plasmid pPR9. Besides *cfr*, resistance genes distributed in the cargo regions included *blaZ*, *lsa*(B), *msr*(A) and *aad*, and a gene cluster for resistance to heavy metals. A closely related *cfr* plasmid (pSP01.1, ~49 kb), differing from pSP01 by the lack of a large cargo region with some resistance genes, was detected in strain SP2.

Conclusions: The conjugative multiresistance plasmid pSP01 is the first *cfr*-carrying plasmid to be sequenced in Italy. This is the first time *cfr* has been found: (i) in association with *blaZ*, *msr*(A) and heavy metal resistance genes; and (ii) in an *S. epidermidis* strain (SP2) belonging to ST83.

Introduction

Linezolid was the first member of the oxazolidinones, a class of synthetic antibiotics introduced into clinical use in the early 2000s for treatment of serious infections by resistant Gram-positive pathogens including methicillin-resistant staphylococci and VRE. Linezolid inhibits protein synthesis by binding to the peptidyl transferase centre of the bacterial ribosome.[1]

Acquired resistance to linezolid emerged shortly after introduction of the drug in clinical practice.[2] Initially, the reported resistance mechanisms were confined to ribosomal alterations, by mutations involving either the 23S rRNA or ribosomal proteins L3 and L4.[3–5] Subsequently, transferable resistance determinants to linezolid have also emerged, including the *cfr* gene[6] and, most recently, the *optrA* gene.[7]

The multiresistance gene *cfr* encodes an rRNA methyltransferase that catalyses post-transcriptional methylation of the C-8 position of nucleotide A2503 in 23S rRNA, and also inhibits ribose methylation at nucleotide C2498.[8] The result is co-resistance to phenicols, lincosamides, oxazolidinones, pleuromutilins and streptogramin A antibiotics ($PhLOPS_A$ phenotype)[9] and reduced susceptibility to 16-membered macrolides.[10]

A vast literature confirms a recent, worldwide spread of *cfr*-harbouring pathogens causing human infections, though with a still low prevalence.[11–14] The potential for dissemination is underscored by the frequent location of *cfr* on mobile genetic elements, typically plasmids, which are important vehicles for its spread, also among bacteria of different species and genera.[6] Dissemination of *cfr* may also be favoured by other factors: (i) the insertion sequences next to which *cfr* is often located;[15,16] (ii) the

low fitness cost of its acquisition;[17] and (iii) its possible co-selection by the various $PhLOPS_A$ agents.

After characterization of pSCFS1, the first identified *cfr*-carrying plasmid from *Staphylococcus sciuri*,[18] *cfr* has been identified in a variety of other plasmids and less frequently in chromosomal DNA. To date, some 30 different *cfr*-carrying plasmids have been reported, differing in size and structure; some have been fully or partially sequenced, and in most of them the immediate genetic environment of *cfr* has been analysed and shown to encode transposases and mobilization proteins, although in variable contexts.[6] However, only a small number of these plasmids have actually been characterized as conjugative plasmids.

In Italy, *cfr*-mediated linezolid resistance has been reported in coagulase-negative staphylococci in the last few years.[19–22] In these studies, the plasmid location of *cfr* was tested and demonstrated only in some cases, but *cfr*-carrying plasmids were not further explored.

In this study, we analysed two linezolid-resistant, *cfr*-positive clinical isolates of *Staphylococcus epidermidis* and investigated the genetic context and transferability of the *cfr* gene.

Materials and methods

Bacterial strains

The two *S. epidermidis* investigated in this work were isolated in 2010 from two inpatients at Florence's Careggi University Hospital, Italy. They were responsible for sporadic cases of bloodstream infections with no apparent epidemiological relationship to each other (the patients were from different wards, in different periods). The strains were resistant to linezolid and, to our best knowledge, were the first *cfr*-positive pathogens detected from that hospital. Further data on these strains are reported in Table 1.

Susceptibility tests

MICs of various antibiotics, all from Sigma Chemical Co., St Louis, MO, USA, were determined by standard broth microdilution.

Amplification experiments

PCR assays were conducted to identify acquired resistance genes or mutations, to sequence unknown DNA segments (inverse PCR) and to map plasmids. All primer pairs used are reported in Table S1 (available as Supplementary data at *JAC* Online).

Besides *cfr*, a number of genetic determinants potentially associated with *Staphylococcus* spp. were sought, encoding resistance to chloramphenicol/florfenicol (cat_{pC194}, cat_{pC221}, cat_{pC223} and *fexA*), macrolides [*erm*(A), *erm*(B), *erm*(C) and *msr*(A)] and β-lactams (*blaZ* and *mecA*).

Testing for linezolid resistance mutations

In each strain, the presence of mutations in domain V of the 23S rRNA gene (23S rDNA) was investigated as previously described,[20] whereas mutations in *rplC* and *rplD* genes, encoding the L3 and L4 ribosomal proteins, respectively, were detected by sequencing the amplicons yielded by primers rplC-FW/rplD-RV (Table S1).

Molecular typing

PFGE analysis of genomic DNA was performed as previously described,[23] with the addition of 50 mg/L lysostaphin (Sigma).

MLST was carried out as previously described,[24] and STs were assigned from the MLST database (http://sepidermidis.mlst.net/).

Table 1. Isolation data, typing data, antimicrobial susceptibility profiles and resistance markers associated with *S. epidermidis* isolates SP1 and SP2

Isolate	Isolation data		Typing		MIC (mg/L)								Genetic resistance markers[a]							
	date	source	pulsotype	ST	LZD	CHL	FFC	CLI	ERY	PEN	OXA	VAN	TET	*cfr*	23S rRNA	L3[b]	cat_{pC221}	*erm*(A)	*msr*(A)	*blaZ*	*mecA*
SP1	summer 2010	blood	A	23	128	128	>128	>128	8	128	64	1	0.25	+	C2534U	A157R	+	+	+	+	+
SP2	spring 2010	central venous catheter	B	83	8	32	>128	>128	0.25	128	64	1	0.5	+	–	–	–	–	–	+	+

LZD, linezolid; CHL, chloramphenicol; FFC, florfenicol; CLI, clindamycin; ERY, erythromycin; PEN, penicillin; OXA, oxacillin; VAN, vancomycin; TET, tetracycline.
[a]Both isolates were negative for other linezolid resistance mutations (G2576T and L4 ribosomal protein), macrolide resistance genes *erm*(B) and *erm*(C), florfenicol resistance gene *fexA* and chloramphenicol resistance genes cat_{pC194} and cat_{pC223}.
[b]A, alanine; R, arginine.

Plasmid isolation

Total plasmid DNA was isolated from the *S. epidermidis* strains using the GeneElute Plasmid Miniprep Kit (Sigma), with the addition of 0.1 mg/L lysostaphin and 1 g/L lysozyme (Sigma) to the buffer. S1-PFGE was used for preliminary assessment of plasmid size.[25]

Southern blotting and hybridization

Plasmid DNA electrophoresis, transfer to nylon membrane, hybridization to biotin-labelled specific probes and probe detection were performed by standard methods. Probes were obtained by PCR with the primer pairs reported in Table S1.

Transformation and mating experiments

Purified plasmids extracted from the two staphylococcal strains were transformed into *Staphylococcus aureus* recipient RN4220 by electrotransformation.[26] Transformants were selected on plates supplemented with florfenicol (10 mg/L).

In mating experiments, *S. epidermidis* strains SP1 and SP2 were used as donors. Recipients were *S. aureus* SA500, a clinical strain susceptible to linezolid (MIC, 0.25 mg/L) and florfenicol (MIC, 2 mg/L), and *Enterococcus faecalis* JH2-2, both selected in our laboratory for streptomycin resistance (MIC, >500 mg/L). Conjugal transfer was performed on a membrane filter. For transconjugant selection, plates supplemented with florfenicol (10 mg/L) and streptomycin (Sigma) (500 mg/L) were used. The frequency of transfer was expressed as the number of transconjugants per recipient.

DNA sequencing, sequence analysis and restriction experiments

DNA sequencing (Sanger approach) and sequence analysis were performed as described elsewhere.[27]

Restriction assays with endonucleases PvuII, EcoRI and XbaI (Roche Applied Science, Basel, Switzerland) were used to analyse and compare amplicons, obtained from PCR mapping, covering the entire *cfr*-carrying plasmid from isolate SP2.

Nucleotide sequence accession number

The complete nucleotide sequence of *cfr*-carrying plasmid pSP01 from *S. epidermidis* SP1 has been submitted to GenBank under accession number KR230047.

Results

Antibiotic susceptibilities and molecular typing

The two *S. epidermidis* investigated in this work were resistant to linezolid, chloramphenicol, florfenicol and clindamycin, in agreement with carriage of the *cfr* gene. The linezolid MIC, however, was higher for SP1 (128 mg/L) than for SP2 (8 mg/L). Both strains were penicillin and oxacillin resistant; SP1 was also erythromycin resistant, while both were susceptible to vancomycin and tetracycline (Table 1).

SP1 and SP2 exhibited different PFGE patterns (data not shown) and belonged to different clonal lineages by MLST analysis (ST23 and ST83, respectively).

Antibiotic resistance determinants

Besides *cfr*, both *S. epidermidis* strains harboured *blaZ* and *mecA*. SP1 also harboured cat_{pC221}, *erm*(A) and *msr*(A). The other resistance genes screened for by PCR were not detected.

Both strains showed WT sequences of the *rplD* gene, whereas a mutation in the *rplC* gene resulting in the amino acid substitution A157R[4] was detected in SP1. The analysis of 23S rDNA revealed a C2534T mutation in SP1 (the 23S rDNA sequence was WT in SP2). The presence of the above mutations was consistent with the higher linezolid MIC for SP1 compared with SP2.

Southern blotting and hybridization with specific probes demonstrated a plasmid location of the *cfr* gene in both isolates, while cat_{pC221}, detected in SP1, hybridized to a plasmid band different from that targeted by the *cfr*-specific probe. *mecA*, detected in both strains, and *erm*(A), detected in SP1, exhibited a chromosomal location.

Transferability of cfr and other resistance genes

To confirm the location of *cfr*, the plasmid preparations from SP1 and SP2 were used to transform *S. aureus* RN4220. MICs of the various antibiotics and PCR data confirmed *cfr* and *blaZ* acquisition by *S. aureus* transformants, and that the transformants obtained with the plasmid preparation from SP1 had also acquired the *msr*(A) gene (Table 2). S1-PFGE and hybridization analysis of transformants revealed a size of ~75 and ~50 kb for *cfr* plasmids acquired from SP1 and SP2 donors, respectively (data not shown).

Table 2. Linezolid, chloramphenicol, florfenicol, erythromycin and penicillin MICs and antibiotic resistance genotypes for *S. epidermidis* donors and relevant transformants

	MIC (mg/L)					Antibiotic resistance genes				
Strain	LZD	CHL	FFC	ERY	PEN	*cfr*	cat_{pC221}	*erm*(A)	*msr*(A)	*blaZ*
S. epidermidis SP1	128	128	>128	8	128	+	+	+	+	+
S. aureus RN4220 TS[a]	8	64	>128	16	2	+	−	−	+	+
S. epidermidis SP2	8	32	>128	0.25	128	+	−	−	−	+
S. aureus RN4220 TS[a]	8	64	128	0.25	1	+	−	−	−	+
S. aureus RN4220	2	4	4	0.25	0.25	−	−	−	−	−

LZD, linezolid; CHL, chloramphenicol; FFC, florfenicol; ERY, erythromycin; PEN, penicillin.
[a]Relevant transformant.

The transferability of *cfr* and other determinants was evaluated by filter-mating experiments. *cfr* was successfully transferred from both *S. epidermidis* donors to *S. aureus* SA500 (frequency, 2.8×10^{-8} for SP1 and 4.5×10^{-6} for SP2), but not to *E. faecalis* JH2-2. MICs and PCR analysis confirmed that the *S. aureus* transconjugants had acquired *cfr* and *blaZ*, as well as *msr*(A) when the donor was strain SP1.

Characterization of cfr plasmid pSP01 from S. epidermidis SP1

The *cfr* plasmid from strain SP1, designated pSP01, was sequenced (accession number KR230047). It was a circular molecule of 76991 bp, with a G+C content of 30% and containing 73 ORFs.

pSP01 displayed a mosaic structure consisting of two backbone regions (G+C content, 29%) and four cargo regions. The genetic map of pSP01 is shown in Figure 1(a) and the major ORF characteristics are detailed in Table S2.

The two backbone regions (22 and 10 ORFs) had high-level identity (95%) and complete synteny to the 32-ORF backbone of conjugative plasmid pPR9 (accession number NC_013653), conferring penicillin and mupirocin resistance in *S. aureus* PR9.[28] Backbones closely related to pPR9 have also been found in two recently described *cfr*-carrying staphylococcal plasmids: p12-00322 from *S. epidermidis* (accession number KM521836)[14] and pLRSA417 from *S. aureus* (accession number KJ922127).[29]

The four cargo regions were distinguished on the basis of their G+C content and similarity to other cargo gene clusters, and included:

(i) *Region of transposases (19406–25268 bp, G+C content 26%)*

This region, which lacked drug resistance genes, contained four genes: three (*orf23*–*orf25*) encoding transposases related (99%–100% DNA identity) to the Tn*554*-like *tnpA*, *tnpB* and *tnpC*, respectively, of *S. epidermidis* plasmid SAP045A (accession number GQ900402); and the last (*orf26*) encoding a hypothetical protein.

(ii) *Genetic context of the blaZ gene (25269–28507 bp, G+C content 28%)*

This region consisted of the *bla* gene complex, i.e. *blaZ* (*orf27*), *blaR1* (*orf28*) and *blaI* (*orf29*), corresponding to the first three ORFs of Tn*552* of pPR9.

(iii) *Genetic context of the cfr gene (28508–39036 bp, G+C content 36%)*

This region included eight ORFs. After *orf30*, there was the clindamycin exporter gene *lsa*(B) (*orf31*), followed by *istAS* (*orf32*) and *istBS* (*orf33*), encoding transposases belonging to the IS*21* family,[15] and the *cfr* gene (*orf34*). The last three ORFs of this region (*orf35*, *orf36* and *orf37*) encoded a resolvase, a transposase and a hypothetical protein, respectively. BLASTN analysis disclosed high-level nucleotide identities to the *cfr* genetic context described in two other plasmids: p12-00322 from *S. epidermidis* (accession number KM521836)[14] and pJP2 from *Staphylococcus rostri* (accession number KC989517).[16] A comparison of the three genetic contexts is illustrated in Figure 1(b).

(iv) *Region bracketed by two IS257-like elements (49300–76991 bp, G+C content 30%)*

This was the largest and most complex cargo region, bounded by two identical IS*257*-like transposase genes, each flanked by 16 bp inverted repeats (Figure S1), having the same direction (*orf48* and *orf73*). The region was inserted between *orf47* and *orf1* (*parM* and *parR* in pPR9, encoding plasmid segregation proteins), and was composed of three different cargo segments, which showed the highest DNA identities to $pETB_{TY825}$ (accession number AP012467), pSE12228-04 (accession number NC_005005) and SAP110A (accession number NC_013383), respectively (Figure S1). The first cargo segment included the resistance gene *msr*(A) (*orf51*). Other resistance genes were detected in the third cargo segment of this region: *aad* (*orf62*), encoding an N6′-acetyltransferase causing aminoglycoside resistance, and a gene cluster (*orf65*, *orf66*, *orf68* and *orf69*) involved in heavy metal (zinc, copper and cobalt) resistance (Table S2).

Characterization of cfr plasmid pSP01.1 from S. epidermidis SP2

PCR mapping of this plasmid was performed using six primer pairs (Table S1), designed from the pSP01 sequence (Figure 1a). Amplicons of the expected sizes were obtained using the four primer pairs covering the two backbone regions and the first three cargo regions of pSP01. These four amplicons were indistinguishable from those yielded by pSP01 in restriction assays. Conversely, no amplification was obtained using primer pairs parM-FW/ATPase-FW3 and rep-FW/hth-FW (Table S1), covering the fourth cargo region of pSP01. However, an ~6 kb product was obtained with primers parM-FW/traE-RV, targeting two pSP01 backbone genes located upstream and downstream of this region. Sequencing of this product confirmed the absence of the fourth cargo region of pSP01. The pSP01-related *cfr* plasmid of *S. epidermidis* SP2 was designated pSP01.1 (estimated size ~49 kb after PCR mapping) (Figure S2).

Discussion

Among staphylococci of clinical origin harbouring the *cfr* gene, *S. epidermidis* is the most relevant species and, although chromosomal location of *cfr* has been reported, a plasmid location of the gene is the most common finding in these isolates.[6]

pSP01 is the first sequenced *cfr*-carrying plasmid from Italy. It exhibits an original mosaic structure with a number of cargo regions inserted into a backbone very similar to that of *S. aureus* plasmid pPR9, which in turn shows extensive structural relatedness to medically important *S. aureus* plasmids of the pSK41/pGO1 family.[28] In spite of their common pPR9-like genetic backbone, pSP01 and the above-mentioned p12-00322[14] and pLRSA417[29] carry a quite diverse set of antibiotic resistance genes, with *cfr* being the only one shared by all three. To our knowledge, this is the first time the *cfr* gene has been found in association with resistance determinants such as *blaZ* and *msr*(A), and heavy metal resistance genes. A distinctive trait of pSP01 is the number and importance of its additional resistance genes, besides *cfr*, which might play a role in *cfr* co-selection and persistence similar to that played by $PhLOPS_A$ drugs.

The ST23 clone—the ST of SP1—is the most prevalent among those detected in *cfr*-positive *S. epidermidis* isolates reported in Italy. This strengthens the notion[21] of a role, in our country, of

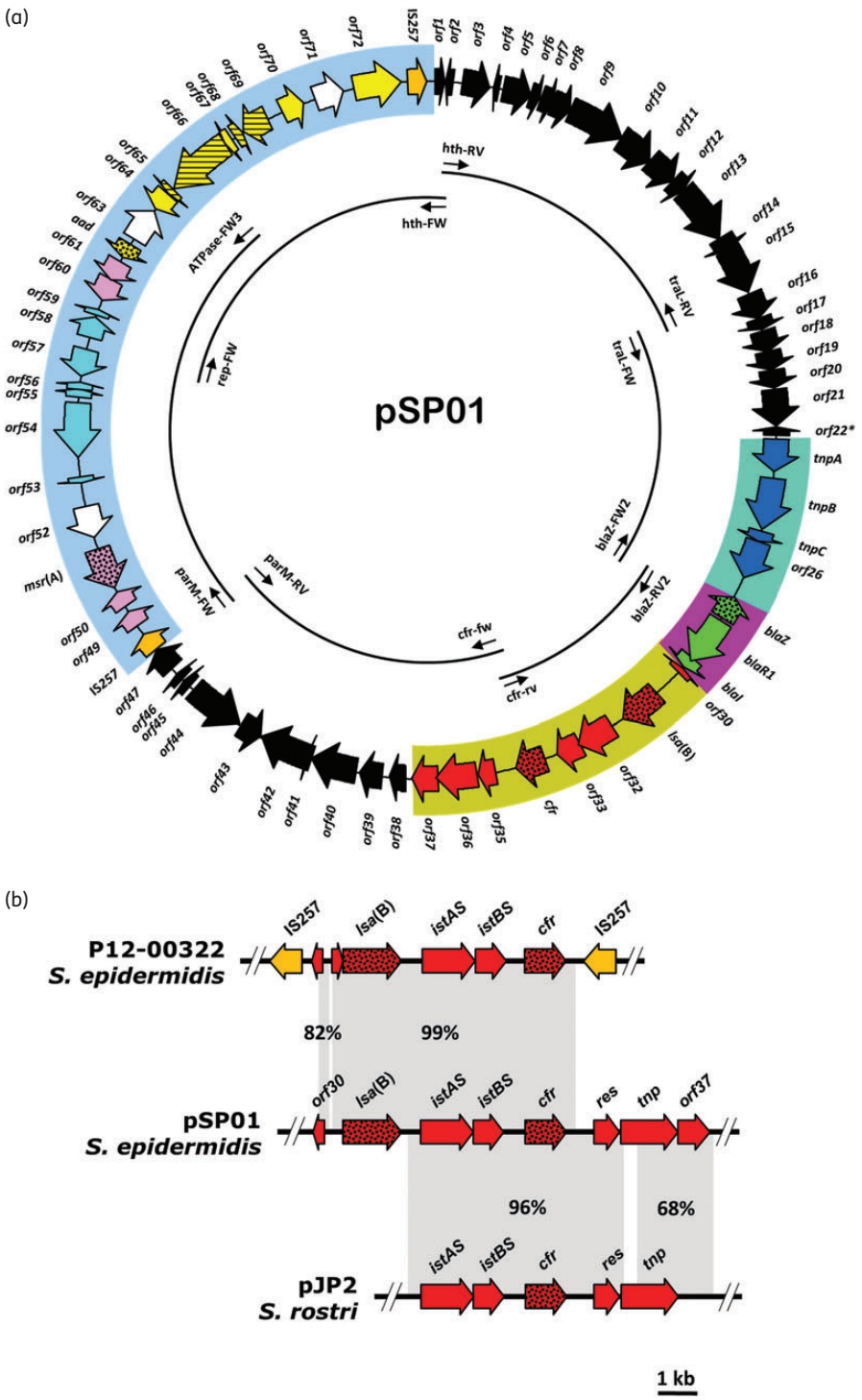

Figure 1. Maps of the *cfr*-carrying plasmid pSP01 from *S. epidermidis* SP1 (76 991 bp) (a) and of the genetic context of *cfr* in this plasmid (b). In panel (a), the 73 ORFs of pSP01 are indicated with their numbers (*orf1*–*orf73*) or names where appropriate. The ORFs of the two backbone regions are represented by black arrows and those of the four cargo regions are represented by arrows of different colours (dotted in the case of antibiotic resistance genes and hatched in the case of genes involved in heavy metal resistance) surrounded by differently coloured areas. Three alien ORFs (*orf52*, *orf63* and *orf71*) are represented by white arrows. The two IS*257*-like transposase genes (*orf48* and *orf73*) bracketing the fourth cargo region are represented by orange arrows. Curved bars inside the circular ORF map indicate the amplicons (each with the relevant primer pair) allowing PCR mapping. In panel (b), the genetic context of *cfr* in plasmid pSP01 is compared with the *cfr* genetic contexts from *S. epidermidis* plasmid p12-00322 (above) and *S. rostri* plasmid pJP2 (below). Grey areas between ORF maps denote percentage DNA identities. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

this genetic background in the acquisition and mobilization of *cfr*-carrying plasmids. Nevertheless, the recent finding of *cfr* plasmids in Italian *S. epidermidis* clinical isolates belonging to ST2[21] and ST83 (SP2 in this study) indicates that further adaptations may subsequently occur. Incidentally, this is the first time that the *cfr* gene has been found in an ST83 *S. epidermidis* strain. This strain carried a closely related variant of plasmid pSP01 (pSP01.1), differing by the lack of an ∼28 kb cargo region, suggesting that the region may be mobile.

cfr-mediated linezolid resistance in coagulase-negative staphylococci may become a concern not only due to their growing clinical relevance, but also because these staphylococci may serve as a *cfr* reservoir for more pathogenic organisms, as suggested by the successful laboratory transfer of pSP01 to an *S. aureus* recipient in the present study.

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Transparency declarations

None to declare.

Supplementary data

Table S1, Table S2, Figure S1 and Figure S2 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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