

Horizontal gene transmission of the cfr gene to MRSA and Enterococcus: role of Staphylococcus epidermidis as a reservoir and alternative pathway for the spread of linezolid resistance

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1 **Horizontal Gene Transmission of *cfr* gene to MRSA and *Enterococcus*:**
2 **role of *S. epidermidis* as reservoir and alternative pathway for the**
3 **spread of linezolid resistance.**
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6 **Fabio Cafini^{1,2,*}, Le Thuy Thi Nguyen^{2,3}, Masato Higashide⁴, Federico Román⁵,**
7 **José Prieto¹, Kazuya Morikawa²**
8

9
10 ¹ Division of Microbiology, Department of Medicine, School of Medicine, Universidad
11 Complutense. Avda. Complutense s/n, 28040 Madrid, Spain
12

13 ² Division of Biomedical Science, Faculty of Medicine, University of Tsukuba, Tsukuba,
14 305-8575, Japan
15

16 ³ Human Biology Program, School of Integrative and Global Majors, University of
17 Tsukuba, 1-1-1 Tennodai, Tsukuba, 305-8577, Japan.
18

19 ⁴ Kotobiken Medical Laboratories, Inc., Kamiyokoba, Tsukuba, 305-0584, Japan
20

21 ⁵ Laboratory of Nosocomial infections, Department of bacteriology, Centro Nacional de
22 Microbiología, Instituto de Salud Carlos III, Majadahonda, Madrid, Spain.
23

24
25
26 *Corresponding author: Fabio Cafini. Division of Microbiology, Department of
27 Medicine, School of Medicine, Universidad Complutense. Avda. Complutense s/n,
28 28040 Madrid, Spain. Tel: +34-91-394-15-12; Fax: +34-91-394-15-11; E-mail:
29 fcafini@ucm.es.
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51 **ABSTRACT**

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53 **Objetives:** Linezolid resistance mediated by *cfr* gene represents a global concern due to
54 its dissemination among multi-resistant nosocomial pathogens such as MRSA and
55 *Enterococcus*. In the present work, we have evaluated the *in vitro* transmission of *cfr*
56 pSCFS7-like plasmids from two *Staphylococcus epidermidis* ST2 strains (SE45 and
57 SE50) isolated in Spanish hospitals, to clinical MRSA and *Enterococcus* spp. isolates
58 obtained in Japan, a country in which *cfr* has not been detected yet. We have also
59 investigated alternative mechanisms of horizontal gene transfer (HGT) involved in the
60 spreading of *cfr* gene.

61 **Methods:** MRSA (n=16) and *Enterococcus* spp. (n=8) clinical isolates were used as
62 recipient in conjugative experiments. Bacteriophage-mediated transmission was tested
63 using MR83a phage and N315, COL and Mu50 strains. Transformation assay was
64 carried out using a natural competent N315 derived strain.

65 **Results:** SE45 strain was able to transfer *cfr* gene to all strains tested, while
66 transmission from SE50 was observed only to a few strains and with less efficiency. No
67 transmission was observed to *Enterococcus* spp. isolates.

68 Even though conjugation is thought to be the main mechanism of *cfr* dissemination, we
69 have demonstrated that transduction can be considered an alternative pathway for the
70 transmission of *cfr* gene between MRSA strains. However, the results suggest an
71 absence of transmission by natural transformation.

72 **Conclusions:** Linezolid resistance mediated by *cfr* vectors, such as pSCFS7-like
73 plasmids, can be efficiently transferred to clinical MRSA in Japanese isolates. After
74 reaching the staphylococcal pool, *cfr* gene could be spread among MRSA strains by
75 either conjugation or transduction.

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98 INTRODUCTION

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100 Linezolid is one of the most active drugs used in the treatment of Gram-positive
101 bacteria. Its activity against MRSA strains, including glycopeptide-resistant ones,
102 makes this molecule an essential clinical tool in the nosocomial-infection therapy.

103 In 2000, a potentially transmissible mechanism of resistance mediated by *cfr* gene
104 (chloramphenicol/florfenicol resistance) was described in one livestock-associated
105 *Staphylococcus sciuri* strain.¹ This gene catalyzes the ribosomal methylation, conferring
106 the PhLOPSA resistance phenotype (Phenicols, Lincosamides, Oxazolidinones,
107 Pleuromutilins, and Streptogramin A antibiotics).^{2,3}

108 Nowadays, the *cfr* gene has been detected in different bacterial species obtained from
109 clinical, livestock and environmental samples.⁴⁻⁷ Even though the presence of this gene
110 only slightly increases the MIC to linezolid in the absence of ribosomal mutations, its
111 ability to be transmitted between different strains or species represents a global concern.
112 This is particularly important in the case of two of the most important Gram-positive
113 nosocomial pathogens, MRSA and *Enterococcus* spp.⁸⁻¹⁰

114 Regarding *Enterococcus* spp., even though the presence of *cfr* in environmental
115 samples has been described,^{6,7} there is only one report in which *cfr* was associated with
116 clinical enterococcal strains.¹⁰ The described strain harbored the *cfr* gene on a ca. 97 kb
117 conjugative plasmid, inserted in a genetic environment identical to the plasmid PSS-01
118 (ca. 54 kb), which was previously detected in livestock-associated staphylococcal
119 isolates in China.¹¹

120

121 The *cfr* gene has been detected in clinical staphylococcal isolates related to hospital
122 outbreaks in different countries of US and Europe.^{12,13} Different plasmids, such as
123 pSCFS3-like plasmids reported in US,¹⁴ pSS-01-like plasmids in Chinese hospitals,¹¹ or
124 pSCFS7-like plasmids in European countries, have been found in staphylococcal
125 strains.^{9,15,16}

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127 The first report of pSCFS7 plasmid was documented in 2010, associated with one
128 clinical Panton-Valentine Leukocidin (PVL)-positive MRSA isolate from Ireland.¹⁷ In
129 this plasmid, *cfr* was related to the Tn558 transposon, inserted in the transposase B gene
130 (*tnpB*) reading frame. In 2012, a linezolid-resistant *S. epidermidis* strain harboring a
131 similar plasmid was detected in Spain.¹⁵ After this report, structurally closely related
132 pSCFS7-like plasmids were further detected associated with clinical infections in Spain
133 and Germany.^{9,16} Regarding the mechanism of dissemination, conjugation has been the
134 only pathway demonstrated for *cfr* transmission. The complete sequence of p12-02300,
135 a pSCFS7-like plasmid obtained from one *S. epidermidis* ST2 isolated in Germany, has
136 been recently described.¹⁶ Even though this kind of vectors appears to be increasingly
137 detected, the described sequence showed an absence of conjugation-associated genes
138 (such as conserved conjugative element *tra/trs* or the associated nickase gene *nes*),
139 suggesting that at least some pSCF7-like plasmids contain non-canonical conjugation
140 system or, alternatively, other horizontal gene transfer (HGT) system. Thus, the
141 detection of plasmids lacking conjugation machinery,¹⁶ together with the strong

142 evidences obtained from clinical transmission of non-conjugative pSCFS3-like
143 plasmids,¹⁴ support the idea that helper machinery or other transmission mechanisms,
144 such as transduction or natural transformation, are involved in the *cfr* dissemination.
145 Although the demonstration of natural transformation in *S. aureus* has been challenging,
146 a cryptic secondary sigma factor *sigH* was found to induce the natural genetic
147 competence for DNA transformation.¹⁸ The artificial overexpression of this factor
148 induces the expression of the *comE* and *comG* operons. These elements encode the
149 DNA uptake machinery, allowing *S. aureus* to develop a competence state and to
150 acquire resistance traits via transformation.

151

152 Coagulase-negative staphylococci (CoNS), especially *S. epidermidis*, have been
153 largely considered to act as genetic reservoir for other pathogenic bacteria. The *cfr*-
154 positive CoNS are increasingly isolated in hospitals due to high consumption of
155 linezolid. This entails the subsequent increase in the *cfr*-positive *S. aureus* and
156 *Enterococcus* spp. strains, which are currently maintained at low frequencies.¹⁹ In Japan,
157 the resistance to linezolid in CoNS and MRSA strains remains hitherto low with no *cfr*-
158 detection in clinical isolates nowadays.²⁰ In this situation, it is important to determine
159 the risk of *cfr* dissemination among clinical isolates in order to prevent the spreading of
160 this resistance.

161 This work aimed to determine the risk of linezolid resistance spreading mediated by
162 the *cfr* gene in Japanese isolates. To that aim, we have measured the ability of two *S.*
163 *epidermidis* strains harboring pSCFS7-like plasmids to transfer the *cfr* gene to different
164 strains belonging to the most important Gram-positive nosocomial pathogens, *S. aureus*
165 and *Enterococcus* spp. In addition, we have studied the HGT-mechanisms involved in
166 the further transmission of this gene from linezolid-resistant *S. aureus* (LRSA) to other
167 *S. aureus* strains.

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186 MATERIALS AND METHODS

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188 Strains used

189 Bacterial strains used in this study are listed in Table S1 (available as Supplementary
190 data at JAC Online). *S. epidermidis* strains were isolated in Spanish hospitals and stored
191 in the reference collection of the Spanish National Center for Microbiology. SE45 was
192 isolated in Madrid and SE50 was isolated in Andalucía region. These strains showed
193 different PFGE profile but belonged to ST 2 group (by Multi-locus sequence type
194 (MLST)). Both strains harbored *cfr* gene on ca. 40 kb plasmids (determined by S1
195 nuclease PFGE hybridization assay) with a pSCFS7 *cfr* insertion region, determined by
196 mapping PCR, (Figure S1-B, available as Supplementary data at JAC Online) using the
197 primers listed in Table S2.

198 MRSA clinical strains (n=16) were isolated in Japan.²¹ Most of the clinical MRSA
199 isolates belonged to Clonal Complex 5 (CC5),²² and all of them harbored SCCmec II
200 type (Table S1).²³

201 *Enterococcus* spp. strains (n=8) were isolated from livestock and human, and they were
202 identified by Api 20 Strep (Sysmex, Japan).

203

204 Molecular analysis of *cfr*-carrying vectors

205 Primers used in the molecular analysis of pSCFS7-like vectors are listed in Table S2.
206 Amplifications were carried out in SE45, SE50 and their N315 *cfr*-positive derivatives
207 (N315-45 and N315-50, see below).

208 Amplification of *traA* and *nes* genes was performed by using primers designed on the
209 basis of pGO1 sequence (accession number FM207042),²⁴ considered the prototype of
210 conjugative staphylococcal plasmid.

211 Primers for backbone amplification of *cfr*-vectors were designed on the basis of the
212 available sequence of pSCFS7 vector p12-02300 (accession number KM521837).¹⁶

213

214 Conjugation

215 Conjugative transmission of *cfr*-associated plasmids was performed using the filter-
216 mating procedure previously described.²⁵

217 Interspecies conjugation: SE45 and SE50 were used as donor strains. MRSA clinical
218 strains (n=16), *Enterococcus* spp. (n=8), N315 and COL reference strains together with
219 their phage cured derivatives (N315ex w/oφ and COL w/oφ)¹⁸ were used as recipient in
220 these experiments.

221 The recipient strains used were chloramphenicol susceptible and erythromycin resistant,
222 except COL strain, which was chloramphenicol susceptible and tetracycline resistant.

223 The susceptibility profile of recipient strains allowed the use of chloramphenicol (32
224 mg/L: to select *cfr* positives) plus erythromycin or tetracycline (32mg/L or 8 mg/L
225 respectively: to eliminate donor *S. epidermidis*) in the selection of transconjugants.
226 Double resistant colonies obtained in interspecies (*S. epidermidis* to MRSA)
227 transmission experiments were confirmed by species assessment (by plating in
228 MacConkey agar) and detection of the *cfr* gene by PCR (Table S2).

229 N315-45 and N315-50 derivatives were obtained by filter mating using as donors SE45

230 and SE50 strains respectively. These *cfr*-positive strains were subsequently used in the
231 characterization of pSCFS7-like vectors.

232

233 MRSA-to-MRSA conjugation: The N315-45, COL-45, N315ex w/o ϕ -45 and COL
234 w/o ϕ -45 *cfr*-positive derivatives (obtained by filter mating using as donor SE45) and their
235 original *cfr*-negative strains were used as donor and recipient respectively in MRSA-to-
236 MRSA transmission.

237 T-N315-45 strain (see below) was also used as donor in these experiments. In addition,
238 a previously described *comG* defective mutant derived from N315ex w/o ϕ (N315ex
239 w/o ϕ Δ *comG*)¹⁸ was used as recipient in MRSA-to-MRSA filter mating experiments.

240 Putative transconjugants obtained in MRSA-to-MRSA transmission were confirmed by
241 assessment of recipient susceptibility profile and detection of the *cfr* gene by PCR.

242

243 **Phage transduction**

244 The bacteriophage MR83a (laboratory stock) is a staphylococcal transducing phage
245 (*Siphoviridae*), suitable for the transduction from/to N315.²⁶

246 First, MR83a was used to infect the N315-45 transconjugant strain. The resultant phage
247 pool (MR83a-45) was harvested and used to test the transmission of *cfr* gene by
248 generalized transduction. 1 mL of the phage pool (2×10^{12} pfu/mL) was mixed with 0.5
249 mL of recipient strains (N315, COL or Mu50), overnight-cultured in nutrient broth (NB,
250 Oxoid) supplemented with 3.6 mM CaCl₂ (NBCaCl₂). Mixture was incubated at 37°C
251 for 30 min. After the addition of 0.1 mL of 20% sodium citrate, the mixture was further
252 incubated for 30 min. Transductants were selected in BHI-agar medium supplemented
253 with chloramphenicol (32 mg/mL).

254 Putative transductants were confirmed by assessment of recipient susceptibility profile
255 and detection of *cfr* gene by PCR. The obtained T-N315-45 *cfr*-positive transductant
256 strain was used to further study the conjugative abilities of *cfr*-positive transductants.

257

258 **Natural transformation**

259 Transformation assay was carried out by using a previously described method.¹⁸ A
260 natural competent strain derivative from N315 (N2-2.1) was used as recipient in these
261 experiments. The N2-2.1 carries a *sigH* locus duplication that constitutively expresses
262 the *sigH* fusion gene, rendering this strain naturally competent.¹⁸

263 Donor DNA in transformation experiments was obtained from the COL-45
264 transconjugant strain. Plasmid fraction (purified by QIAfilter Plasmid Midi kit,
265 QIAGEN) and whole DNA (conventional purification method) were used as source of
266 *cfr* in these experiments.

267 Transformation of pT181 and pHY300PLK plasmids, purified from COL and *E. coli*
268 HST04 respectively, was performed in parallel as positive control, generating
269 transformation frequencies about $10^{-9} \sim 10^{-10}$.¹⁸

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274 **RESULTS**

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276 **Conjugative transmission from *S. epidermidis* to Japanese isolates**

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278 Results obtained in interspecies transmission (*S. epidermidis* to *S. aureus*) are
279 summarized in Figure 1. SE 45 strain was able to transfer *cfr* gene to all strains tested
280 with a mean frequency of $8,57 \times 10^{-5}$ transconjugants per recipient cell. On the contrary,
281 only half of the tested strains showed transmission from SE50, and this transmission
282 took place with a lower efficiency (mean $5,21 \times 10^{-9}$ transconjugant per recipient cell).
283 These results show the existence of differential efficiency of transmission among *S.*
284 *epidermidis* strains.

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286 In the case of *Enterococcus* spp., no *cfr* *in vitro* transmission was observed for any
287 strain tested.

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290 **Molecular analysis of pSCFS7-like vectors**

291

292 We have investigated the existence of the conserved conjugative element (*tra/trs*) and
293 the associated nickase gene *nes* in SE45 and SE50 *cfr*-vectors. Primers (Table S2) were
294 designed on the basis of the published sequences of pGO1²⁴ and the recently described
295 *cfr*-positive pSCFS6-like plasmid.¹⁶ The amplification of *traA* and *nes* genes was tested
296 in SE45, SE50, and in their *cfr*-positive N315-45 and N315-50 derivatives. Negative
297 amplification was obtained in the case of SE45, whereas positive amplification results
298 for both genes were obtained in SE50 strain (data not shown). Negative amplifications
299 were obtained in both N315-45 and N315-50 strains, showing the absence of these
300 genes in SE45 and SE50 pSCFS7-like vectors.

301

302 In order to determine the structural similarity between p12-02300, SE45 and SE50
303 pSCFS7-like plasmids, a set of primers located outside the *cfr* insertion context was
304 designed on the basis of the available p12-02300 sequence (Table S2). Amplifications
305 were tested in SE45, SE50 and N315-45 and N315-50 derivative strains. Amplifications
306 (b1, b2 and b3, Figure S2) gave expected size products in the four strains (data not
307 shown), suggesting the backbone similarities between SE45 and SE50 pSCFS7-like
308 plasmids and p12-02300.

309

310 **MRSA can transfer *cfr* by filter mating in absence of phage and competence**
311 **machinery.**

312

313 As shown in Figure 2A, N315-45 and COL-45 were able to further transfer *cfr* to COL
314 and N315 respectively, showing that these strains retained the transmission ability after
315 they had acquired it from *S. epidermidis*.

316 In order to test the involvement of native phages in this transmission process, a set of

317 MRSA-to-MRSA filter mating experiments were performed in absence of native phages.
318 N315ex w/o ϕ -45 and COL w/o ϕ -45, *cfr*-positive strains, were used as donor, while their
319 original *cfr*-negative were used as recipient strains. In these experiments, a similar
320 transconjugant generation was observed (Figure **2B**), showing that resident phages are
321 not significantly involved in the transmission of this vector by filter mating.

322

323 We also tested the existence of transformation process in filter mating experiments. The
324 *comG* defective mutant derived from N315ex w/o ϕ (N315ex w/o ϕ Δ *comG*) was used as
325 recipient in this transmission experiments. The recipient strain lacked essential genes
326 for competence development (*comG* operon genes), being unable to obtain external
327 DNA by transformation. Using the COL w/o ϕ -45 strain as a donor in MRSA-to-MRSA
328 filter mating, we were able to observe the transmission in the absence of natural
329 transformation machinery. As shown in Figure **2B**, similar transconjugant generation
330 was observed in the case of this recipient strain, suggesting the absence of
331 transformation process in the filter mating experiments.

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334 **Phage mediated transmission of pSCFS7-like plasmids**

335

336 In *S. aureus*, phage transduction is thought to play a major role in HGT, since most of
337 the *S. aureus* isolates are lysogenized. The DNA size which can be packed in this
338 transducing phage is up to 39-43 kbp.²⁷ Thus, we expected that our *cfr* plasmid (c.a. 40
339 kbp) was transferrable by phage transduction. The potential spreading of *cfr* gene by
340 transduction was tested by using staphylococcal transducing phage MR83a (see material
341 and methods). The phage was amplified by infecting the strain N315-45, and its ability
342 to transduce the *cfr* gene was tested in the recipient strains N315, COL and Mu50 (both
343 strains were capable of acquiring *cfr* by filter mating methodology from N315-45, Table
344 **1**). All recipient strains were able to obtain *cfr* by transduction (Table **1**), generating the
345 *cfr*-positive T-N315-45, T-COL-45 and T-Mu50-45 strains. These results showed the
346 bacteriophage-mediated transmission of *cfr* gene between MRSA strains, and, in
347 addition, the potential coexistence of linezolid and vancomycin resistance determinants
348 in Mu50 strain.

349

350 To further investigate the involvement of transduction in the global spreading of *cfr*
351 gene, the T-N315-45 transductant strain was used (Table **1**). This strain was analyzed in
352 order to determine the integrity of pSCFS7-like plasmid after transduction, as well as its
353 conjugative ability. The analysis of the genetic environment of *cfr* insertion and the *cfr*-
354 carrying plasmid backbone showed an indistinguishable amplification pattern for SE45,
355 N315-45 and T-N315-45 strains (Figure **S1** and **S2** respectively), suggesting the
356 transmission of complete pSCFS7-like vector by transduction.

357 In addition, the *cfr* gene was successfully transferred by filter mating from T-N315-45
358 to COL, showing a transconjugant generation similar to the previously observed in the
359 case of transmission from the N315-45 to COL (Figure **2A**). These data suggested the
360 retention of conjugative capability after transduction process.

361 **Acquisition of *cfr* by natural transformation.**

362

363 In the present work, we have also investigated the potential transmission of the *cfr* gene
364 by natural transformation. To that aim, a N315 derivate (N2-2.1)¹⁸ constitutively
365 expressing *sigH* (see material and methods) was used as the recipient strain in
366 transformation experiments.

367 As shown in Table 1, no transformants harboring *cfr* were observed using as donor
368 DNA plasmid fraction or whole genome extraction from COL-45, suggesting a low
369 relevance of transformation in the *cfr* dissemination among MRSA strains.

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405 DISCUSSION

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407 In this work, we have analyzed the risk of *cfr* transmission to a collection of clinical
408 MRSA and *Enterococcus* spp. strains by conjugative transference. As shown in Figure 1,
409 a differential transmission pattern could be observed in the two *S. epidermidis* strains.
410 Whereas SE45 strain is able to transfer *cfr* to all MRSA tested with high frequency,
411 transmission from SE50 was observed only to a few strains and with less efficiency.
412 These results show the existence of differential efficiency of transmission among the *cfr*
413 reservoir, which takes place even in the case of *S. epidermidis* strains belonging to the
414 same MLST group (ST2).

415 Regarding the *cfr* dissemination to *Enterococcus* spp., no transmission was observed for
416 any strain tested. In this regard, the transmission of resistance traits between strains of
417 both genera, such as the transmission of vancomycin resistance through conjugative
418 plasmids, has been previously described.²⁸ In addition, the transmission of *cfr* from *S.*
419 *epidermidis* to *E. faecalis* through conjugative pSCFS6-like plasmids has also been
420 reported,¹⁶ although there is no information regarding the transmission of pSCFS7-like
421 plasmids between these genera.

422 The lack of transmission observed in this work suggests the limitation of the pSCFS7-
423 like plasmids spreading to enterococci. In addition, although the existence of these
424 plasmids has been reported in different staphylococcal species, there is no evidence of
425 clinical *Enterococcus* spp. associated with pSCFS7-like plasmids, not even in countries
426 such as Spain in which these vectors are commonly found.^{9,15} This suggests that the
427 spreading of pSCFS7-like plasmids could be restricted to staphylococci.

428

429 In our work, we studied the presence of *traA* and *nes* genes in SE45, SE50 and in the
430 N315 *cfr*-positive derivate strains (N315-45 and N315-50). The detection of conjugative
431 machinery in SE50 strain but not on its N315-50 derivate suggested that conjugative
432 genes were not associated with pSCFS7-like plasmid, but related to chromosomal or
433 additional native conjugative plasmid in this strain.

434

435 The absence of conjugation-associated machinery in the p12-02300 plasmid sequence
436 suggested the existence of differences between this plasmid and conjugative pSCFS7-
437 like plasmids isolated in Spain.^{9,15} These differences could be related to the existence of
438 mobilization events comprising the pSCFS7 *cfr* genetic environment to different
439 plasmid backbones.¹⁶ In order to investigate this hypothesis, a set of PCR amplifications
440 was performed to test the similarities between p12-02300 and SE45,SE50 vectors.
441 Amplifications gave the expected products in both *S. epidermidis* isolates and N315 *cfr*-
442 positive derivatives, showing the backbone similarities of these pSCFS7-like plasmids
443 and p12-02300.

444 The structural similarities observed in pSCSF7-like plasmids and the absence of
445 canonical conjugative machinery called into question the nature of the transmission of
446 these vectors. To confirm the conjugational nature of the transference of these plasmids,
447 we studied the MRSA-to-MRSA transmission of SE45 pSCFS7-like plasmid in order to
448 rule out potential interferences due to phage transduction or to natural transformation.

449 Strains lacking its native phages and deficient in DNA uptake were able to acquire *cfr*
450 by filter mating with similar frequencies to their unmodified strains, showing the low
451 impact of transduction and transformation in the filter mating model, and how
452 conjugation or certain unknown HGT could act as the main mechanism of transmission
453 of this pSCFS7-like plasmid.

454 Even though the conjugative transmission of *cfr* has been largely demonstrated,^{7-10,15}
455 some aspects of the dissemination phenomenon still remain unclear. Unlike in Europe,
456 in the US, the *cfr* gene has been preferentially found on pSCFS3-like plasmids. These
457 plasmids have been related to clinical isolates and hospital outbreaks,^{13,14} and, although
458 they are considered as non-conjugative on the basis of *in vitro* results, strong evidences
459 indirectly demonstrated their transmission among staphylococci: identical pSCFS3
460 plasmids were found in two strains belonging to different staphylococcal species (CoNS
461 and MRSA).¹⁴ This finding suggested the transmission of *cfr* through the mobilization
462 by helper systems, or, alternatively, the existence of different HGT mechanisms which
463 allowed its spreading among the staphylococcal pool. In this work, we analyzed the
464 involvement of different HGT systems, such as transduction and natural transformation,
465 in the *cfr* transmission.

466
467 Bacteriophage transduction represents a relevant HGT mechanism involved in the
468 genetic plasticity and evolution of *S. aureus*, and it can be considered as the most
469 important mechanism of gene transmission in this bacterial species.²⁹ N315, COL and
470 Mu50 strains were able to acquire *cfr* by transduction. The transductant strains showed
471 an indistinguishable amplification pattern for *cfr* genetic environment and plasmid
472 backbone, compared to the transconjugant or to the original *cfr*-positive *S. epidermidis*
473 strains. Strikingly, its conjugative capability seems to remain active after transduction,
474 rendering transductant strain capable of spreading *cfr* by conjugation.

475
476 The results obtained in this work showed that pSCFS7-like vectors can be efficiently
477 transferred to clinical MRSA in countries in which this gene has not been yet detected
478 such as Japan. These data point to the relevance of surveillance programs aimed to the
479 early detection of *cfr* gene in both MRSA and non-pathogenic bacteria such as CoNS,
480 and especially in *S. epidermidis*, the reservoir of this gene for MRSA strains. In the case
481 of pSCFS7-like plasmids, some reservoirs can transfer *cfr* gene more efficiently than
482 others.

483
484 In addition, we have demonstrated that emerging linezolid-resistant *S. aureus* strains are
485 able to spread *cfr* not only by conjugation, but also, by phage-mediated transduction.
486 This is the first report, to our knowledge, of a conjugation-independent HGT
487 mechanism for *cfr* gene transmission in MRSA strains, and it might provide an answer
488 for the observed dissemination of non-conjugative *cfr*-vehicles, such as pSCFS3-like
489 vectors, detected in other countries.

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499 **TRANSPARENCY DECLARATIONS**

500 None to declare.

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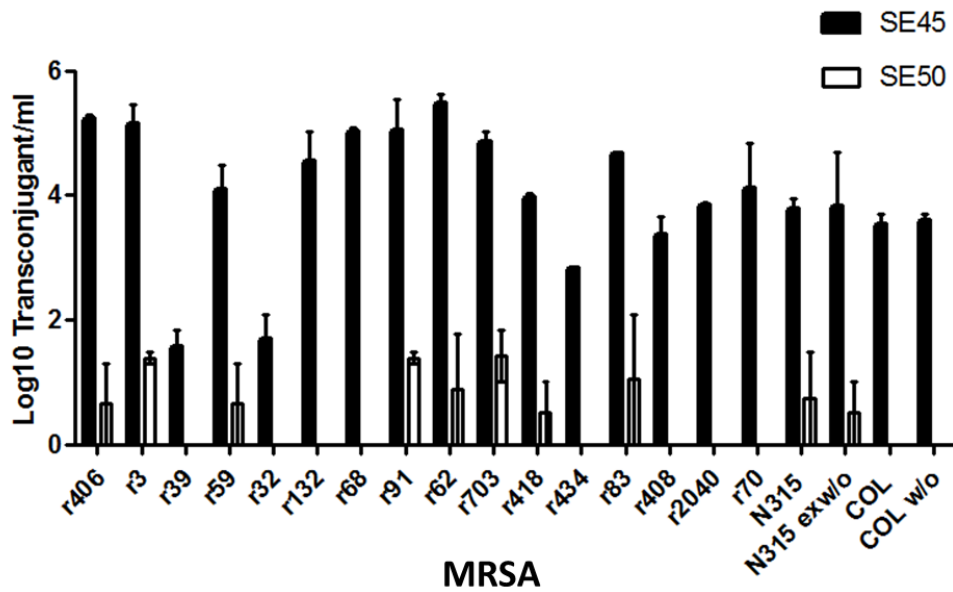
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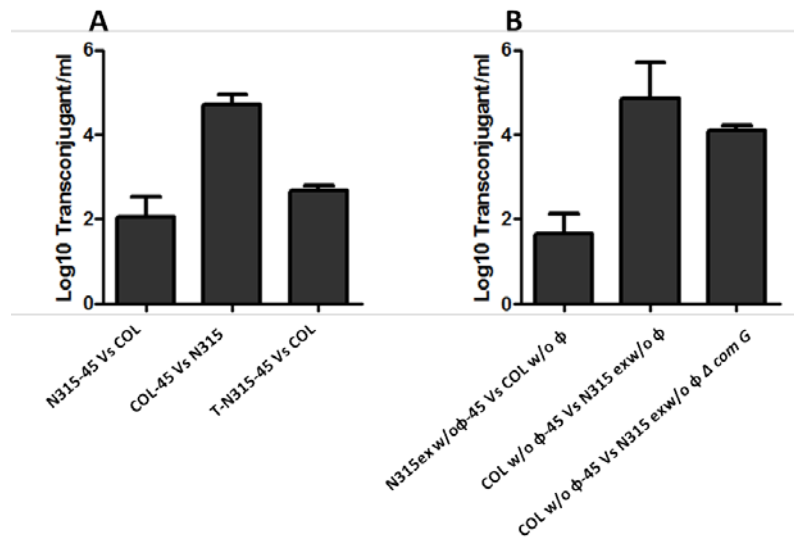
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Figure 1: Representation of transconjugant generation (expressed as Log10/ml) obtained in filter mating experiments by using clinical *cfr*-positive *S. epidermidis* strains (SE45 and SE50) as donor. Clinical MRSA strains (n=16), N315 and COL strains and their phage cured derivatives N315ex w/o ϕ and COL w/o ϕ were used as recipient in these experiments. The represented values correspond to average data obtained from 2 independent experiments. Filled bars represent values of SE45 transmission. Open bars represent values of SE50 transmission



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707 **Figure 2:** Representation of transconjugant generation (expressed in Log10/ml) in MRSA-to-
 708 MRSA filter mating experiments. All donor strains harbored pSCFS7-like plasmids from SE45
 709 strain. **A:** Transmission results obtained using unmodified N315 and COL *cfr*-positive strains.
 710 N315-45 and COL-45 acquired *cfr* by conjugation from SE45. T-N315-45 and T-COL-45
 711 strains acquired *cfr* by transduction from N315-45 through MR83a phage. **B:** Transmission
 712 results obtained using N315 and COL cured phage free derivatives. N315ex w/o ϕ -45 and COL
 713 w/o ϕ -45 acquired *cfr* by conjugation from SE45. N315ex w/o ϕ Δ comG is a DNA uptake
 714 defective mutant derived from N315ex w/o ϕ . The represented values correspond to average data
 715 obtained from 2 independent experiments.

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733 **Table 1:** Investigation of horizontal gene transfer mechanisms involved in the *cfrr* transmission
734 in MRSA strains. The frequency is expressed in transconjugant/recipient cell in conjugation
735 experiments, whereas it is expressed in transductant/plaque forming unit (PFU) in transduction
736 experiments. The represented values correspond to average data obtained from 2 independent
737 experiments. ULD: under limit of detection.

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HTG	Donor	Recipient	Frequency
Conjugation	N315-45	COL	1.00×10^{-6}
	N315-45	Mu50	1.29×10^{-5}
Transduction	N315-45	N315	6.88×10^{-10}
	N315-45	COL	1.00×10^{-11}
	N315-45	Mu50	3.68×10^{-10}
Transformation	Plasmids (COL-45)	N2-2.1	ULD
	Whole DNA (COL-45)	N2-2.1	ULD

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