Horizontal Gene Transmission of *cfr* gene to MRSA and *Enterococcus*: role of *S. epidermidis* as reservoir and alternative pathway for the spread of linezolid resistance.

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ABSTRACT

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

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

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

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

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

INTRODUCTION

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

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

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

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

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

The first report of pSCFS7 plasmid was documented in 2010, associated with one clinical Panton-Valentine Leukocidin (PVL)-positive MRSA isolate from Ireland.[17] In this plasmid, *cfr* was related to the Tn*558* transposon, inserted in the transposase B gene (*tnpB*) reading frame. In 2012, a linezolid-resistant *S. epidermidis* strain harboring a similar plasmid was detected in Spain.[15] After this report, structurally closely related pSCFS7-like plasmids were further detected associated with clinical infections in Spain and Germany.[9, 16] Regarding the mechanism of dissemination, conjugation has been the only pathway demonstrated for *cfr* transmission. The complete sequence of p12-02300, a pSCFS7-like plasmid obtained from one *S. epidermidis* ST2 isolated in Germany, has been recently described.[16] Even though this kind of vectors appears to be increasingly detected, the described sequence showed an absence of conjugation-associated genes (such as conserved conjugative element *tra*/*trs* or the associated nickase gene *nes*), suggesting that at least some pSCF7-like plasmids contain non-canonical conjugation system or, alternatively, other horizontal gene transfer (HGT) system. Thus, the detection of plasmids lacking conjugation machinery,[16] together with the strong

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

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

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

MATERIALS AND METHODS

Strains used

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

MRSA clinical strains (n=16) were isolated in Japan.[21] Most of the clinical MRSA isolates belonged to Clonal Complex 5 (CC5),[22] and all of them harbored SCC*mec* II type (Table **S1**).[23]

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

Molecular analysis of *cfr*-carrying vectors

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

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

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

Conjugation

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

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

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

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

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

MRSA-to-MRSA conjugation: The N315-45, COL-45, N315ex w/oϕ-45 and COL w/oϕ-45 *cfr*-positive derivates (obtained by filter mating using as donor SE45) and their original *cfr*-negative strains were used as donor and recipient respectively in MRSA-to-MRSA transmission.
T-N315-45 strain (see below) was also used as donor in these experiments. In addition, a previously described *comG* defective mutant derived from N315ex w/oϕ (N315ex w/oϕ *ΔcomG*)[18] was used as recipient in MRSA-to-MRSA filter mating experiments.
Putative transconjugants obtained in MRSA-to-MRSA transmission were confirmed by assessment of recipient susceptibility profile and detection of the *cfr* gene by PCR.

Phage transduction

The bacteriophage MR83a (laboratory stock) is a staphylococcal transducing phage (*Siphoviridae*), suitable for the transduction from/to N315. [26]
First, MR83a was used to infect the N315-45 transconjugant strain. The resultant phage pool (MR83a-45) was harvested and used to test the transmission of *cfr* gene by generalized transduction. 1 mL of the phage pool (2×10^{12} pfu/mL) was mixed with 0.5 mL of recipient strains (N315, COL or Mu50), overnight-cultured in nutrient broth (NB, Oxoid) supplemented with 3.6 mM $CaCl_2$ ($NBCaCl_2$). Mixture was incubated at 37ºC for 30 min. After the addition of 0.1 mL of 20% sodium citrate, the mixture was further incubated for 30 min. Transductants were selected in BHI-agar medium supplemented with chloramphenicol (32 mg/mL).
Putative transductants were confirmed by assessment of recipient susceptibility profile and detection of *cfr* gene by PCR. The obtained T-N315-45 *cfr*-positive transductant strain was used to further study the conjugative abilities of *cfr*-positive transductants.

Natural transformation

Transformation assay was carried out by using a previously described method.[18] A natural competent strain derivate from N315 (N2-2.1) was used as recipient in these experiments. The N2-2.1 carries a *sigH* locus duplication that constitutively expresses the *sigH* fusion gene, rendering this strain naturally competent. [18]
Donor DNA in transformation experiments was obtained from the COL-45 transconjugant strain. Plasmid fraction (purified by QIAfilter Plasmid Midi kit, QIAGEN) and whole DNA (conventional purification method) were used as source of *cfr* in these experiments.
Transformation of pT181 and pHY300PLK plasmids, purified from COL and *E. coli* HST04 respectively, was performed in parallel as positive control, generating transformation frequencies about $10^{-9} \sim 10^{-10}$.[18]

RESULTS

Conjugative transmission from *S. epidermidis* to Japanese isolates

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

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

Molecular analysis of pSCFS7-like vectors

We have investigated the existence of the conserved conjugative element (*tra*/*trs*) and the associated nickase gene *nes* in SE45 and SE50 *cfr*-vectors. Primers (Table **S2**) were designed on the basis of the published sequences of pGO1[24] and the recently described *cfr*-positive pSCFS6-like plasmid.[16] The amplification of *traA* and *nes* genes was tested in SE45, SE50, and in their *cfr*-positive N315-45 and N315-50 derivates. Negative amplification was obtained in the case of SE45, whereas positive amplification results for both genes were obtained in SE50 strain (data not shown). Negative amplifications were obtained in both N315-45 and N315-50 strains, showing the absence of these genes in SE45 and SE50 pSCFS7-like vectors.

In order to determine the structural similarity between p12-02300, SE45 and SE50 pSCFS7-like plasmids, a set of primers located outside the *cfr* insertion context was designed on the basis of the available p12-02300 sequence (Table **S2**). Amplifications were tested in SE45, SE50 and N315-45 and N315-50 derivate strains. Amplifications (b1, b2 and b3, Figure **S2**) gave expected size products in the four strains (data no shown), suggesting the backbone similarities between SE45 and SE50 pSCFS7-like plasmids and p12-02300.

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

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

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

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

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

Phage mediated transmission of pSCFS7-like plasmids

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

To further investigate the involvement of transduction in the global spreading of *cfr* gene, the T-N315-45 transductant strain was used (Table **1**). This strain was analyzed in order to determine the integrity of pSCFS7-like plasmid after transduction, as well as its conjugative ability. The analysis of the genetic environment of *cfr* insertion and the *cfr*-carrying plasmid backbone showed an indistinguishable amplification pattern for SE45, N315-45 and T-N315-45 strains (Figure **S1** and **S2** respectively), suggesting the transmission of complete pSCFS7-like vector by transduction.
In addition, the *cfr* gene was successfully transferred by filter mating from T-N315-45 to COL, showing a transconjugant generation similar to the previously observed in the case of transmission from the N315-45 to COL (Figure **2A**). These data suggested the retention of conjugative capability after transduction process.

Acquisition of *cfr* by natural transformation.

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

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

DISCUSSION

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

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

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

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

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

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

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

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

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

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

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

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TRANSPARENCY DECLARATIONS

None to declare.

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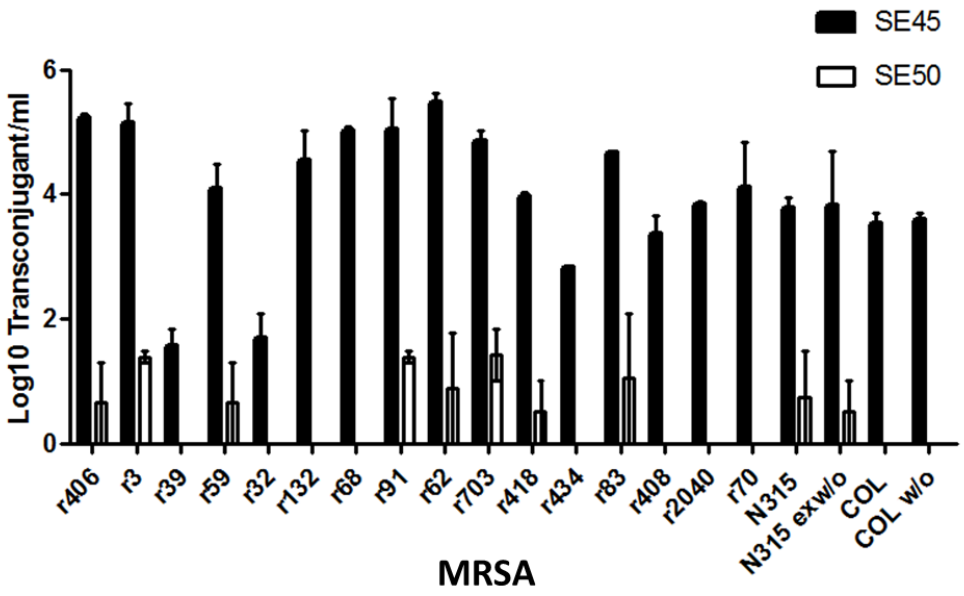

Figure 1: Representation of transconjugant generation (expressed as Log10/ml) obtained in filter mating experiments by using clinical *cfr*-positive *S. epidermidis* strains (SE45 and SE50) as donor. Clinical MRSA strains (n=16), N315 and COL strains and their phage cured derivates N315ex w/oϕ and COL w/oϕ 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

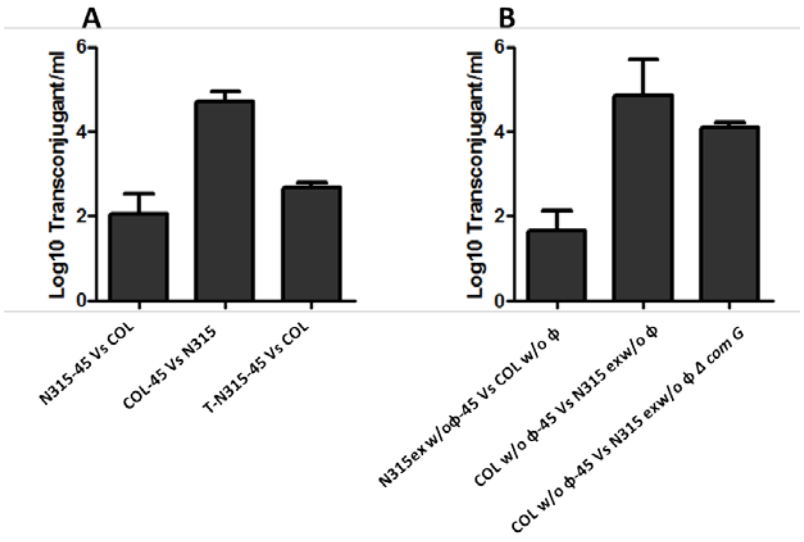

Figure 2: Representation of transconjugant generation (expressed in Log10/ml) in MRSA-to-MRSA filter mating experiments. All donor strains harbored pSCFS7-like plasmids from SE45 strain. **A:** Transmission results obtained using unmodified N315 and COL *cfr*-positive strains. N315-45 and COL-45 acquired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

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

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