Mechanisms of linezolid resistance among clinical *Staphylococcus* spp. in Spain. Spread of methicillin- and linezolid-resistant *S. epidermidis* ST2

Running title: Linezolid resistance among clinical staphylococci

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Abstract

This study aimed at determining the mechanisms of linezolid resistance and the molecular characteristics of clinical *Staphylococcus aureus* (n=2) and coagulase-negative staphylococci (n=15) isolates obtained from four Spanish hospitals. The detection of linezolid resistance mechanisms (mutations and acquisition of resistance genes) was performed by PCR/sequencing. The antimicrobial resistance and virulence profile was determined, and the isolates were typed by different molecular techniques. Moreover, the genetic environment of the *cfr* gene was determined by whole genome sequencing. The *cfr* gene was detected in one methicillin-resistant *S. aureus* (MRSA) that also displayed the amino acid change Val118Ala in the ribosomal protein L4. The second *S. aureus* isolate was methicillin-susceptible and showed different alterations in the ribosomal protein L4. All remaining linezolid-resistant *S. epidermidis* (n=14) and *S. hominis* isolates (n=1) showed the mutation G2576T (n=14) or C2534T (n=1) in the 23S rRNA. Moreover, different amino acid changes were detected in the ribosomal proteins L3 and L4 in *S. epidermidis* isolates. All *S. epidermidis* isolates belonged to the multi-locus sequence type ST2. Linezolid resistant staphylococci (LRS) showed a multiresistance phenotype, including methicillin resistance that was detected in all isolates but one, and was mediated by the *mecA* gene. The *cfr* gene in the MRSA isolate was located together with the *fexA* gene on a conjugative 38,864 bp plasmid. Linezolid- and methicillin-resistant *S. epidermidis* ST2 showing mutations in the 23S rRNA and in the ribosomal proteins L3 and L4 are spread among Spanish hospitals, whereas LRS carrying acquired linezolid resistance genes are rarely detected.

Keywords: linezolid; *S. epidermidis*; ST2; *S. aureus*; *cfr*

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-resistant coagulase-negative staphylococci (MRCoNS) are important pathogens involved in community- and hospital-associated infections. Frequently, these bacteria are not only resistant to β-lactam antibiotics, but also to several other classes of antimicrobial agents.[1,2] This fact, in addition to the capacity of certain staphylococcal species, such as *S. epidermidis*, to produce biofilms, compromise the therapeutic success.[2]

In this context, linezolid is the first member of the oxazolidinone class of antimicrobial agents, which has demonstrated good efficacy against multiresistant Gram-positive pathogens, including MRSA and MRCoNS.[1,3,4] Nearly two decades after its introduction into clinical use, linezolid remains active against approximately 99% of Gram-positive bacteria.[5]

In *Staphylococcus* spp., the main mechanism of linezolid resistance involves point mutations in the central loop of domain V of the 23S rRNA. Moreover, decreased susceptibility to linezolid has also been related to amino acid changes and alterations in the ribosomal proteins L3 (*rplC*), L4 (*rplD*) and L22 (*rplV*).[1,4-6] However, linezolid resistance mediated by acquired resistance genes is concerning because of its great capacity of dissemination. Three transferable linezolid resistance genes have been detected in staphylococci so far. The *cfr* gene mediates resistance to five classes of antimicrobial agents (phenicols, lincosamides, oxazolidinones, pleuromutilins, and streptogramin A) leading to a multiresistance phenotype. Since its first detection in *S. sciuri*,[7,8] *cfr* has been reported in several Gram-positive and Gram-negative bacteria of diverse origins.[9] More recently, the *optrA* and *poxtA* genes were described in *Enterococcus* spp. and *S. aureus*, respectively. Both genes confer reduced susceptibility to oxazolidinones as well as to phenicols. Additionally, the *poxtA* gene decreases the tetracycline susceptibility.[10,11]

Given the importance of linezolid as a last resort antimicrobial agent in human medicine, it is critical to assess the currently molecular mechanisms of resistance and especially to evaluate the presence of the abovementioned linezolid resistance genes in the clinical setting. Therefore, the objective of the present study was to identify the mechanisms of linezolid resistance, and to study the molecular characteristics of linezolid-resistant staphylococci (LRS) recovered from four Spanish hospitals located in different regions.

Materials and methods

Bacterial collection

During the period 2017-2019, LRS exhibiting a minimum inhibitory concentration (MIC) of > 4 mg/L to linezolid,[12] as confirmed by E-test (bioMérieux, Durham, USA), were recovered from four Spanish hospitals located in four different geographic regions. The hospitals that took part in this study are the following: Hospital San Pedro (Logroño), Hospital Royo-Villanova (Zaragoza), Hospital Verge de la Cinta (Tortosa), and Hospital Arnau Vilanova (Lleida).

Isolates of each patient that belonged to different staphylococcal species and/or showed different antimicrobial resistance phenotypes were characterized. These included 14 *S. epidermidis*, two *S. aureus*, and one *S. hominis,* recovered from 15 different patients. Three isolates were obtained from the same patient: *S. epidermidis* X316 and *S. hominis* X315 were recovered at the same time point, and *S. epidermidis* X507 one week later. LRS were recovered from blood (n=7), nasal/pharyngeal samples (n=3), and other sources (n=7) (Table 1).

Antimicrobial resistance pheno- and genotypes

The susceptibility to penicillin, oxacillin, ampicillin, erythromycin, clindamycin, gentamicin, tobramycin, streptomycin, tetracycline, ciprofloxacin, levofloxacin, vancomycin, teicoplanin, daptomycin, fosfomycin, mupirocin, fusidic acid, and trimethoprim-sulfamethoxazole was studied using the MicroScan® WalkAway (Beckman Coulter, Brea, USA). The MIC to rifampicin, chloramphenicol, and florfenicol was measured by broth macrodilution for the *cfr*-positive isolate, using *S. aureus* ATCC 29213 as quality control. The CLSI standard M100[12] was used to evaluate the MIC results of all antimicrobial agents, except fosfomycin, mupirocin, fusidic acid,[13] and streptomycin,[14] for which the methods and breakpoints recommended by the Committée de l'Antibiogramme de la Société Française de Microbiologie were employed.

Isolates were PCR-screened for the presence of the linezolid resistance genes *cfr*, *optrA*, and *poxtA* (Supplementary Table S1). Moreover, mutations in 23S rRNA were investigated by PCR and amplicon sequencing, and by digestion with the *Nhe*I restriction enzyme.[15] The presence of amino acid changes in the genes encoding the ribosomal proteins L3 (*rplC*), L4 (*rplD*), and L22 (*rplV*) were determined in all isolates by PCR and sequencing (Supplementary Table S1). The obtained sequences were compared with those of linezolid-susceptible *S. aureus* NCTC 8325 (GenBank accession number CP000253) and *S. epidermidis* ATCC 12228 (GenBank accession number CP022247) using the EMBOSS Needle software for nucleotide or amino acid (BLOSUM 62 cost matrix) alignments. The sequence chromatogram of the 23S rRNA, *rplC*, *rplD*, and *rplV* were carefully checked in order to avoid false-positive observations.

According to the antimicrobial resistance phenotype, the presence of the antimicrobial resistance genes *blaZ*, *mecA*, *mecB*, *mecC*, *erm*(A), *erm*(B), *erm*(C), *msr*(A), *mph*(C), *lnu*(A), *lnu*(B), *lsa*(B), *vga*(A), *aac*(6')-Ie-*aph*(2")-Ia, *ant*(4')-Ia, *str*, *ant*(6)-Ia, *tet*(L), *tet*(M), *tet*(K), *vanA*, *vanB*, *mupA*, *fusB*, *fusC*, *dfrA*, *dfrD*, *dfrG*, and *dfrK* was studied by PCR

(Supplementary Table S1). Positive controls from the collection of the University of La Rioja were included in all PCR assays.

In the isolates that showed resistance to fluoroquinolones, amino acid changes in the deduced sequences of GyrA and GrlA proteins were investigated by PCR and sequencing, and compared with the wild-type reference strains *S. aureus* NCTC 8325 (GenBank accession number CP000253) and *S. epidermidis* ATCC 12228 (GenBank accession number CP022247) (Supplementary Table S1).

Molecular typing

Characterization by *spa* typing was performed in *S. aureus* isolates by PCR and sequencing, and the obtained sequences were analyzed using Ridom Staph-Type© software (Ridom GmbH, Münster, Germany). *S. aureus* and *S. epidermidis* isolates were subjected to MLST (Multilocus Sequence Typing). In addition, methicillin-resistant staphylococci were characterized by SCC*mec* (Staphylococcal Cassette Chromosome *mec*) typing (Supplementary Table S1).

Virulence gene content

The presence of the genes encoding the virulence factors Panton-Valentine leucocidin (PVL) (*lukF*/*S*-PV), toxic-shock syndrome toxin (*tst*) and exfoliative toxins (*eta*, *etb*, and *etd*) was investigated in all staphylococcal isolates by PCR. The five genes (*scn*, *chp*, *sak*, *sea*, and *sep*) that comprise the IEC (Immune Evasion Cluster) system were investigated in *S. aureus* isolates (Supplementary Table S1).

Whole-Genome-Sequencing (WGS) and genetic environment of the *cfr* gene

The genetic context of the *cfr* gene was determined by WGS. The DNA extraction was performed using the QIAamp® DNA Mini Kit (QIAGEN, Hilden, Germany) with modifications. Before starting the protocol, the cells were mixed with 25 µL lysostaphin

solution (0.1 mg/mL) and incubated for 25 min at 37°C. After that, 75 µL TE buffer and 25 µL proteinase K (0.1 mg/L) were added and incubated for 25 min at 37°C. Then, 75 µL PBS and 2 µL RNAse (2 µg/µL) were added and slightly mixed. After this, the protocol for the kit was followed starting with the addition of AL buffer. The libraries for WGS were prepared using the Nextera XT library preparation kit (Illumina Inc., San Diego, USA) according to the manufacturer's instructions. The 2×300 bp paired-end sequencing in 40-fold multiplexes was performed on the Illumina MiSeq platform (Illumina Inc., San Diego, USA). Genome sequences were *de novo* assembled using the software MIRA 4.0 (Biomatters, Auckland, New Zealand) and annotated using RAST.[16] The nucleotide sequences were analyzed using Geneious v 2019.0.4 (Biomatters, Auckland, New Zealand), and with the online tools ResFinder[17] and VirulenceFinder[18] of the Center for Genomic Epidemiology website. Nucleotide alignments were performed using Geneious alignment with default settings and amino acid alignments with the BLOSUM62 cost matrix.

A set of primers (F-CCTTGTAAGTTGTGAAACAAACACA and R-AGTCTAAATGGCTTTCATCTGCTTT) was designed to complete the *cfr*-carrying plasmid based on the sequence of *Staphylococcus epidermidis* strain 12-02300 plasmid p12-02300 (GenBank accession number KM521837).

Conjugation experiments

Conjugation experiments were performed to evaluate the transfer of the *cfr* gene by the filter-mating method[19] using a rifampicin-resistant mutant of the *S. aureus* ATCC® 29213 as recipient strain. The selection of the transconjugants was performed using three different strategies: (1) Selection on Brain Heart Infusion (BHI) agar containing 50 mg/L rifampicin and 10 mg/L chloramphenicol, (2) Selection on BHI agar containing 50 mg/L rifampicin and 10 mg/L florfenicol, and (3) Selection on BHI agar containing 50 mg/L rifampicin and 4 mg/L linezolid. The identification of the transconjugants was performed by *spa* typing

(Supplementary Table S1). MICs of the transconjugants to clindamycin, chloramphenicol, florfenicol, and linezolid[12] were determined by broth macrodilution and their respective resistance genotype was determined by PCR (Supplementary Table S1).

Results

Mechanisms of linezolid resistance

The detailed characteristics of the LRS investigated in this study are shown in Table 1. The isolates showed different combinations of linezolid resistance mechanisms. The single MRSA isolate (linezolid MIC 16 mg/L) harbored the multiresistance gene *cfr* and the amino acid change Val118Ala in the ribosomal protein L4. The methicillin-susceptible *S. aureus* (MSSA) isolate showed a two amino acid deletion at positions 66 and 67 and the amino acid change Val118Ala in the ribosomal protein L4 with respect to the wild-type sequence. All linezolid-resistant *S. epidermidis* (LRSE, n=14) displayed the highest linezolid MICs identified in this study (MICs >256 mg/L). Mutations within the domain V of the 23S rRNA gene were detected in all cases: G2576T (n=13) and C2534T (n=1). Different amino acid changes and insertions were found in the deduced sequences of the ribosomal proteins L3 and/or L4 among *S. epidermidis* isolates, while all the isolates were wild-type for L22 (Table 1). The *S. hominis* isolate, which showed a linezolid MIC of 32 mg/L, also showed the mutation G2576T in the 23S rRNA nucleotide sequence. Neither *optrA* nor *poxtA* genes were detected among the LRS.

Molecular typing, resistance to other antimicrobial agents and virulence gene content

The MRSA and the MSSA isolates were typed as t2220-ST125 and t1688-ST123, respectively, and all LRSE were assigned to the sequence type ST2 (Table 1). All LRS showed a multiresistance phenotype (resistance to three or more classes of antimicrobial

194 agents). All LRS, except one *S. aureus*, were methicillin-resistant and carried the *mecA* gene
195 whereas *mecB* and *mecC* genes were not detected. The SCC*mec* type IV was identified in the
196 MRSA isolate, whereas all methicillin-resistant *S. epidermidis* carried the SCC*mec* type III.
197 The *S. hominis* isolate carried a non-typeable SCC*mec* element. Thirteen LRS isolates
198 displayed macrolide and/or lincosamide resistance, which was mediated by *erm*(A), *erm*(C),
199 *msr*(A), *lnu*(A), and/or *vga*(A) genes. Resistance to at least one aminoglycoside was detected
200 in all LRS with the exception of the MSSA isolate. The aminoglycoside resistance genes
201 *aac*(6')-Ie-*aph*(2'')-Ia, *ant*(4')-Ia, *aph*(3')-III, *str*, and/or *ant*(6)-Ia genes were detected (Table
202 1). The MSSA and one *S. epidermidis* isolate displayed resistance to tetracycline and the
203 *tet*(L) and *tet*(K) genes were detected, respectively. Fluoroquinolone resistance, seen in all
204 MRCoNS, and in the MRSA isolate was mediated by amino acid changes in the deduced
205 sequences of the GyrA (Ser84Leu, Ser84Tyr, Ser84Phe, Glu88Lys, and/or Ala457Thr) and
206 the GrlA (Ser80Phe, Asp84Tyr, Gly84Asp, and/or Tyr410Phe) proteins. The *fexA* gene was
207 found in the MRSA isolate which displayed MICs to chloramphenicol and florfenicol of 128
208 mg/L and 512 mg/L, respectively. The *mupA* gene was detected in four out of the seven
209 mupirocin-resistant isolates. All *S. epidermidis* isolates showed resistance to fusidic acid,
210 which was mediated by the *fusB* gene. The *dfrA* (n=14) and *dfrG* (n=1) genes were
211 responsible of the resistance to trimethoprim detected in all MRCoNS. One *S. epidermidis*
212 isolate was classified as intermediate to vancomycin, but neither *vanA* nor *vanB* resistance
213 genes were detected.

214 None of the virulence associated genes tested (*lukS/F*-PV, *tst*, *eta*, *etb* and *etd*) were
215 detected among our isolates. The MSSA isolate carried the *scn* and *sak* genes, and therefore
216 was ascribed to IEC type E. The WGS allowed us to identify the presence of different
217 virulence genes in the MRSA isolate, including hemolysins (*hlgA*, *hlgB*, *hlgC*), leucotoxins
218 (*lukD*, *lukE*), aureolysin (*aur*), enterotoxins (*seg*), and proteases (*splA*, *splB*).

Genetic environment of the *cfr* gene and conjugation assays

The analysis of the whole genome sequence of isolate *S. aureus* C9026 identified the *cfr* gene as located in combination with the *fexA* gene on a 38,864 bp plasmid. A very similar plasmid as the one detected in this study, was previously described in the S*taphylococcus epidermidis* strain 12-02300 (GenBank accession number KM521837).[20] The only difference between the plasmid described in the present study and the p12-02300 was the amino acid change Asp184Tyr in one hypothetical protein. The plasmid carrying the *cfr* and *fexA* genes was successfully transferred into *S. aureus* ATCC® 29213 using linezolid for selection of transconjugants. The transconjugants displayed resistance to clindamycin (32 mg/L), chloramphenicol (128 mg/L), florfenicol (256 mg/L), and linezolid (8 mg/L) (Table 2). Apart from *cfr* and *fexA*, no other resistance genes were detected in the transconjugants.

Discussion

When linezolid was introduced in 2000, it was thought that, due to its unique mechanism of action and not being structurally related to other known family of antimicrobial agents, it would be difficult for bacteria to develop resistance.[3] However, one year after its introduction, the first linezolid-resistant *S. aureus* isolate showing the G2576T mutation in the 23S rRNA was reported.[21] Since then, several studies have described the emergence of linezolid resistance among *Staphylococcus* spp. worldwide.[1,6,20,22-28]

Previous studies have reported the presence of the *poxtA* gene in staphylococci recovered from humans,[11,29] whereas, to the best of our knowledge, the *optrA* gene has not been described in clinical LRS. In this study, we have only detected the presence of the *cfr* gene in one MRSA isolate. This gene has been reported in clinical LRS in different

242 countries,[5,20,22,25,27] and, as in our study, it has been previously described to co-occur with
243 other mechanisms of linezolid resistance.[20,25]

244 As also observed by other authors,[5,25,27] changes in different target sites associated
245 with oxazolidinone resistance have become common among LRS. Several studies have
246 demonstrated that the main mechanism of linezolid resistance among *Staphylococcus* spp. is
247 attributable to point mutations in the 23S rRNA and, as in our study, the change G2576T is
248 the most commonly detected worldwide.[5,6,20,24,25,27] The mutation C2534T that displayed one
249 *S. epidermidis* isolate of this study has been previously detected among clinical isolates of this
250 species.[30] Several of the non-synonymous mutations in the gene encoding the ribosomal
251 protein L3 detected in this study (e.g., Val154Leu, Ala157Arg, Met156Thr, Leu101Val) have
252 been previously reported among clinical linezolid-resistant *S. epidermidis* isolates.[5,6,25,27,30]
253 However, no data exist regarding other amino acid changes, such as Gly137Val. As it was
254 observed in several studies, the gene encoding the ribosomal protein L4 seems to have more
255 probable insertions and deletions,[5,6,30] and the insertion of a glycine at position 71 detected in
256 seven *S. epidermidis* isolates in this study, has been previously described.[5,6] Conversely,
257 amino acid changes in the ribosomal protein L22 remain uncommon among LRS.[5,6,25,27] The
258 association between some of these amino acid changes in the ribosomal proteins L3 and L4,
259 and the decreased susceptibility to linezolid has not yet been found, since other works have
260 reported changes (e.g., Leu101Val) which do not seem to be involved in linezolid
261 susceptibility.[22,24,28]

262 Among CoNS, *S. epidermidis* is the most clinically relevant species, primarily
263 associated with foreign body-related infections.[2] Linezolid resistance has been previously
264 detected in *S. epidermidis* isolates of different STs (e.g., ST22, ST5, ST23, ST24, ST185,
265 ST186),[30,31] but the most prevalent one is ST2. *S. epidermidis* belonging to ST2 (clonal
266 complex CC5) is the most important genetic lineage related to hospital-associated infections

267 and involved in linezolid resistance worldwide.[2,5,22,24] Becker *et al*.[2] attributed the spread of
268 this clonal type in clinical settings to its capacity to produce biofilm and the presence of
269 numerous resistance genes. In the case of Spain, linezolid-resistant *S. epidermidis* of ST2
270 have been previously described in hospitals of the same and different regions where the
271 hospitals that took part in this work are located.[23,26, 27]

272 In this study, linezolid resistance was associated with a multiresistance phenotype in
273 all cases, and with methicillin-resistance in all but one isolate, which is in accordance with
274 previous works.[2,6,25] As it was observed by other authors, resistance to vancomycin,
275 teicoplanin and daptomycin is uncommon among both linezolid-resistant CoNS and *S.*
276 *aureus*. However, LRS frequently display resistance to macrolides and lincosamides,
277 gentamicin, and fluoroquinolones.[6,25]

278 Regarding virulence associated genes, none of the CoNS isolates carried any of the
279 ones studied. The virulence genes investigated are strongly associated with *S. aureus* but,
280 although few studies do exist about their presence among CoNS, they have been previously
281 detected in different species of diverse origins.[32-35] The MSSA isolate harbored the
282 staphylococcal complement inhibitor (*scn*) and the staphylokinase (*sak*) genes, and so, it was
283 ascribed to the IEC type E. The IEC facilitates human colonization and invasion and,
284 therefore, its detection is common in lineages adapted to humans.[36] The MRSA isolate
285 harbored several genes which are major contributors to *S. aureus* virulence. Some of these
286 virulence associated genes such as *lukDE*, *aur*, and the hemolysin genes are frequently
287 detected in *S. aureus* isolates of diverse origins.[37,38]

288 In this study, we have detected a *cfr*- and *fexA*-carrying plasmid in one MRSA isolate.
289 The genetic structure containing the *cfr* and *fexA* genes, including the transposases *tnpA*, *tnpB*,
290 and *tnpC*, is similar to the Tn*558* variant described in the plasmid pSCFS7 of a clinical
291 MRSA strain (GenBank accession number FR675942)[39]. The pSCFS7-like plasmids have

been reported many times in different staphylococcal species in European countries, including Spain.[40,41] Moreover, the first *cfr*-carrying *S. epidermidis* isolate in Spain belonged to the lineage ST22 and also harbored the *cfr* and *fexA* genes in a similar structure to pSCFS7.[31] Although, the whole genome sequence analysis did not reveal the presence of known conjugation-associated machinery in the *cfr*-MRSA isolate detected in our study, this plasmid was transferred by conjugation. However, the horizontal gene transmission of pSCFS7-like plasmids by conjugation, even in absence of conjugation machinery, has been previously reported.[42]

In conclusion, linezolid-resistant *S. epidermidis* ST2 with mutations in different oxazolidinone target sites are present in Spanish hospitals. LRS, carrying acquired linezolid resistance genes, are uncommon in isolates recovered from humans. LRS showed a multiresistance phenotype, but remained susceptible to some last resort antimicrobial agents, such as daptomycin. These results highlight the need for continued epidemiological surveillance in order to better understand the characteristics of LRS.

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316 **Author Disclosure Statement**

317 No competing financial interests exist.
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Table 1. Characterization of the seventeen linezolid-resistant *Staphylococcus* spp. recovered from four Spanish hospitals.

Strain	Species	Hospital[a]	Sample type	MLST (*spa* type)	SCC*mec*	Linezolid MIC (mg/L)	Mechanism of linezolid resistance[b]	Antimicrobial resistance phenotype[c]	Antimicrobial resistance genotype	Virulence genes
C9026	*S. aureus*	HRV	Pharyngeal	ST125 (t2220)	IV	16	*cfr*; L4 (Val118Ala)	OXA-PEN-AMP-ERY-CLI-STR-CIP[d,h,j,m]-LEV-CHL-FFN-LZD-FOS	*mecA*, *blaZ*, *msr*(A), *aph*(3')-III, *ant*(6)-Ia, ***cfr*,** *fexA*, *fosD*	*hlgA*, *hlgB*, *hlgC*, *lukD*, *lukE*, *seg*, *aur*, *splA*, *splB*
C9906	*S. aureus*	HSP	Sputum	ST123 (t1688)	-	8	L4 (del-Lys66Gln67, Val118Ala)	PEN-AMP-TET-LZD	*blaZ*, *tet*(L)	IEC type E[n]
C10354	*S. epidermidis*	HRV	Nasal	ST2	III	>256	23S rRNA (G2576T); L3 (Leu101Val, Val154Leu)	OXA-PEN-AMP-ERY-CLI-GEN-TOB-STR-TET-CIP[e,g,j,k]-LEV-LZD-VAN[I]-FOS-MUP-FUS-SXT	*mecA, blaZ, erm*(C), *aac*(6')-Ie-a*ph*(2'')-Ia, *str, tet*(K), *fusB*, *dfrA*	
C10356	*S. epidermidis*	HRV	Pharyngeal	ST2	III	>256	23S rRNA (G2576T); L3 (Leu101Val, Gln136Leu, Met156Thr); L4 (Gly69Arg, in-70Gly71)	OXA-PEN-AMP-ERY-CLI-GEN-TOB-CIP[e,j,k]-LEV-LZD-FUS-SXT	*mecA*, *blaZ*, *erm*(C), *msr*(A), *aac*(6')-Ie-*aph*(2'')-Ia, *ant*(4')-Ia, *str*, *fusB*, *dfrA*	
X466	*S. epidermidis*	HRV	Skin	ST2	III	>256	23S rRNA (G2576T); L3 (Leu101Val, Val154Leu, Met156Thr); L4 (in-70Gly71)	OXA-PEN-AMP-ERY-CLI-GEN-TOB-CIP[e,j,k]-LEV-LZD-MUP-FUS-SXT	*mecA, erm*(C), *aac*(6')-Ie-a*ph*(2'')-Ia, *fusB*, *dfrA*	
C10515	*S. epidermidis*	HSP	Blood	ST2	III	>256	23S rRNA (C2534T); L3 (Leu101Val, Ala157Arg); L4 (Asn217Ser)	OXA-PEN-AMP-ERY-CLI-GEN-TOB-STR CIP[f,g,h]-LEV-LZD-FUS-SXT	*mecA*, *blaZ*, *erm*(C), *aac*(6')-Ie-*aph*(2'')-Ia, a*nt*(4')-Ia, *str*, *fusB*, *dfrA*	
C10040	*S. epidermidis*	HSP	Dialysis catheter	ST2	III	>256	23S rRNA (G2576T); L3 (Ala83Val, Leu101Val, Gly139Val, His146Arg, Met156Thr)	OXA-PEN-AMP-GEN-TOB- CIP[e,j,k]-LEV-LZD-FUS-SXT	*mecA, aac*(6')-Ie-a*ph*(2'')-Ia, *fusB*, *dfrA*	

X530	*S. epidermidis*	HSP	Blood	ST2	III	>256	23S rRNA (G2576T); L3 (Leu101Val, Gln136Leu, Met156Thr); L4 (Gly69Arg, in-70Gly71)	OXA-PEN-AMP-ERY-CLI-GEN-TOB-CIP[e,j,k]-LEV-LZD-MUP-FUS-SXT	*mecA*, *blaZ*, *erm*(C), *aac*(6')-Ie-*aph*(2'')-Ia, *ant*(4')-Ia, *mupA*, *fusB*, *dfrA*
X544	*S. epidermidis*	HSP	Blood	ST2	III	>256	23S rRNA (G2576T); L3 (Leu101Val, Gln136Leu, Met156Thr); L4 (in-70Gly71)	OXA-PEN-AMP-GEN-TOB-CIP[e,j,k]-LEV-LZD-FUS-SXT	*mecA*, *blaZ*, *aac*(6')-Ie-*aph*(2'')-Ia, *ant*(4')-Ia, *fusB*, *dfrA*
X548	*S. epidermidis*	HSP	Urine	ST2	III	>256	23S rRNA (G2576T); L3 (Leu101Val, Gln136Leu, Met156Thr)	OXA-PEN-AMP-CLI-GEN-TOB-CIP[e,j,k]-LEV-LZD-MUP-FUS-SXT	*mecA*, *blaZ*, *vga*(A), *aac*(6')-Ie-*aph*(2'')-Ia, *ant*(4')-Ia, *mupA*, *fusB*, *dfrA*
X507	*S. epidermidis*	HSP	Blood	ST2	III	>256	23S rRNA (G2576T); L3 (Leu101Val, Gln136Leu, Met156Thr)	OXA-PEN-AMP-ERY-CLI-GEN-TOB-CIP[e,j,k]-LEV-LZD-MUP-FUS-SXT	*mecA*, *blaZ*, *erm*(C), *aac*(6')-Ie-*aph*(2'')-Ia, *ant*(4')-Ia, *mupA*, *fusB*, *dfrA*
X316	*S. epidermidis*	HSP	Blood	ST2	III	>256	23S rRNA (G2576T); L3 (Leu101Val, Gly137Val, His146Arg, Met156Thr)	OXA-PEN-AMP-CLI-GEN-TOB-CIP[e,j,k]-LEV-LZD-FUS-SXT	*mecA, blaZ, vga*(A), *aac*(6')-Ie-*aph*(2'')-Ia, *fusB*, *dfrA*
X529	*S. epidermidis*	HVC	Blood	ST2	III	>256	23S rRNA (G2576T); L3 (Leu101Val, Gly139Val, His146Arg, Met156Thr)	OXA-PEN-AMP-ERY-CLI-GEN-TOB-CIP[e,j,k]-LEV-LZD-TEI-FUS-SXT	*mecA*, *blaZ, erm*(C), *aac*(6')-Ie-a*ph*(2'')-Ia, *fusB*, *dfrA*
X1758	*S. epidermidis*	HAV	Wound exudate	ST2	III	>256	23S rRNA (G2576T); L3 (Leu101Val, Phe147Leu); L4 (in-70Gly71)	OXA-PEN-AMP-CLI-GEN-TOB-CIP[e,j,k]-LEV-LZD-MUP-FUS-SXT	*mecA*, *blaZ*, *vga*(A), *aac*(6')-Ie-a*ph*(2'')-Ia, *fusB, dfrA*
X1759	*S. epidermidis*	HAV	Venous catheter	ST2	III	>256	23S rRNA (G2576T); L3 (Leu101Val, Gln136Leu, Met156Thr, Ser214Leu); L4 (in-70Gly71)	OXA-PEN-AMP-ERY-CLI-GEN-TOB-CIP[e,j,k]-LEV-LZD-FUS-SXT	*mecA*, *blaZ, erm*(A), *erm*(C), *vga*(A), *aac*(6')-Ie-a*ph*(2'')-Ia, *fusB, dfrA*

X1760	*S. epidermidis*	HAV	Venous catheter	ST2	III	>256	23S rRNA (G2576T); L3 (Leu101Val, Gln136Leu, Met156Thr); L4 (in-70Gly71)	OXA-PEN-AMP-CLI-GEN-TOB-CIP[e,j,k]-LEV-LZD-FUS-SXT	*mecA*, *blaZ, vga*(A), *aac*(6')-Ie-a*ph*(2'')-Ia, *fusB, dfrA*
X315	*S. hominis*	HSP	Blood	-	Non-typeable	32	23S rRNA (G2576T)	OXA-PEN-AMP-ERY-CLI-GEN-TOB-CIP[f,j,l]-LEV-LZD-TEI-MUP-SXT	*mecA*, *blaZ*, *erm*(C), *lnu*(A), *aac*(6')-Ie-*aph*(2'')-Ia, *ant*(4')-Ia, *mupA*, *dfrG*

[a]HSP, Hospital San Pedro; HRV, Hospital Royo Villanova; HAV, Hospital Arnau Villanova: HVC, Hospital Verge de la Cinta.
[b]In brackets the mutations, amino acid changes, deletions (del), or insertions (in) detected.
[c]OXA, oxacillin; PEN, penicillin; AMP, ampicillin; ERY, erythromycin; CLI, clindamycin; GEN, gentamicin; TOB, tobramycin; STR, streptomycin; TET, tetracycline; CIP, ciprofloxacin; LEV, levofloxacin; VAN, vancomycin; CHL, chloramphenicol; FFN, florfenicol; LZD, linezolid; TEI, teicoplanin; FOS, fosfomycin; MUP, mupirocin; FUS, fusidic acid; SXT, trimethoprim-sulfamethoxazole. I, intermediate resistance.
[d]Ser84Leu in GyrA protein.
[e]Ser84Tyr in GyrA protein.
[f]Ser84Phe in GyrA protein.
[g]Glu88Lys in GyrA protein.
[h]Ala457Thr in GyrA protein.
[j]Ser80Phe in GrlA protein.
[k]Asp84Tyr in GrlA protein.
[l]Gly84Asp in GrlA protein.
[m]Tyr410Phe in GrlA protein.
[n]Immune Evasion Cluster (IEC) type E contains *scn* and *sak* genes.

Table 2. Minimum inhibitory concentrations (MICs) of the recipient *S. aureus* ATCC® 29213, the donor *S. aureus* C9026, and the transconjugant C9026-TC isolate.

Isolate	MIC (mg/L)			
	Clindamycin	Chloramphenicol	Florfenicol	Linezolid
***S. aureus* ATCC® 29213**	0.12	4	4	2
C9026	64	128	512	16
C9026-TC	32	128	256	8

Table S1. Primer pairs used for the characterization of linezolid-resistant staphylococci.

Target gene	Primer Sequence (5'-3')	Amplicon size (bp)	Reference
Multilocus sequence typing *S. aureus*			
arcC	F: TTGATTCACCAGCGCGTATTGTC	456	1
	R: AGGTATCTGCTTCAATCAGCG		
aroE	F: ATCGGAAATCCTATTTCACATTC	456	
	R: GGTGTTGTATTAATAACGATATC		
glpF	F: CTAGGAACTGCAATCTTAATCC	465	
	R: TGGTAAAATCGCATGTCCAATTC		
gmk	F: ATCGTTTTATCGGGACCATC	429	
	R: TCATTAACTACAACGTAATCGTA		
pta	F: GTTAAAATCGTATTACCTGAAGG	474	
	R: GACCCTTTTGTTGAAAAGCTTAA		
tpi	F: TCGTTCATTCTGAACGTCGTGAA	402	
	R: TTTGCACCTTCTAACAATTGTAC		
yqiL	F: CAGCATACAGGACACCTATTGGC	516	
	R: CGTTGAGGAATCGATACTGGAAC		
Multilocus sequence typing *S. epidermidis*			
arcC	F: TGTGATGAGCACGCTACCGTTAG	465	2
	R: TCCAAGTAAACCCATCGGTCTG		
aroE	F: CATTGGATTACCTCTTTGTTCAGC	420	

	R: CAAGCGAAATCTGTTGGGG		
gtr	F: CAGCCAATTCTTTTATGACTTTT	438	
	R: GTGATTAAAGGTATTGATTTGAAT		
mutS	F: GATATAAGAATAAGGGTTGTGAA	412	
	R: GTAATCGTCTCAGTTATCATGTT		
pyr	F: GTTACTAATACTTTTGCTGTGTTT	428	
	R: GTAGAATGTAAAGAGACTAAAATGAA		
tpi	F: ATCCAATTAGACGCTTTAGTAAC	424	
	R: TTAATGATGCGCCACCTACA		
yqiL	F: CACGCATAGTATTAGCTGAAG	416	
	R: CTAATGCCTTCATCTTGAGAAATAA		
***spa*-typing**			
spa	F: AGACGATCCTTCGGTGAGC	Variable	3
	R: GCTTTTGCAATGTCATTTACTG		
SCC*mec*-typing			
ccrA2-ccrB	F: TAAAGGCATCAATGCACAAACACT	937	4
	R: ATTGCCTTGATAATAGCCITCT		
ccrA3-ccrB	F: AGCTCAAAAGCAAGCAATAGAAT	1791	
	R: ATTGCCTTGATAATAGCCITCT		
mecA-IS*1272*	F: ATGCTTAATGATAGCATCCGAATG	2827	
	R: ATATACCAAACCCGACAACTACA		
mecA-mecI	F: CATAACTTCCCATTCTGCAGATG	1963	
	R: ATATACCAAACCCGACAACTACA		
Resistance genes			

optrA	F:AGGTGGTCAGCGAACTAA R: ATCA ACTGTTCCCATTCA	1395	5
poxtA	F: TCAATGCAGAGCAGGAAGCA R: GGTGGATTTACCGACACCGT	791	6
cfr	F: TGAAGTATAAAGCAGGTT GGGAGTCA R: ACCATATAATTGACCACA AGCAGC	746	7
blaZ	F: CAGTTCACATGCCAAAGAG R: TACACTCTTGGCGGTTTC	772	8
mecA	F: GGGATCATAGCGTCATTATTC R: AACGATTGTGACACGATAGCC	527	9
mecB	F: TTAACATATACACCCGCTTG R: TAAAGTTCATTAGGCACCTCC	527	10
mecC	F: GCTCCTAATGCTAATGCA R: TAAGCAATAATGACTACC	304	11
erm(A)	F: TCTAAAAAGCATGTAAAAGAA R: CTTCGATAGTTTATTAATATTAG	645	12
erm(B)	F: GAAAAGTACTCAACCAAATA R: AGTAACGGTACTTAAATTGTTTA	639	
erm(C)	F: TCAAAACATAATATAGATAAA R: GCAAATATTGTTTAAATCGTCAAT	642	
msr(A)	F: GGCACAATAAGAGTGTTTAA AGG R: AAGTTATATCATGAATAGATTGTCCTGTT	399	13

mph(C)	F: ATGACTCGACATAATGAAAT R: CTACTCTTTCATACCTAACTC	900	8
lnu(A)	F: GGTGGCTGGGGGGTAGATGTATTAACTGG R: GCTTCTTTTGAAATACATGGTATTTTTCGATC	322	14
lnu(B)	F: CCTACCTATTGTTTGTGGAA R: ATAACGTTACTCTCCTATTC	944	15
lsa(B)	F: TGCCGAAGCCATGTACCGTCC R: CGGTTAGACCAACCAGCCGAACG	396	16
vga(A)	F: AGTGGTGGTGAAGTAACACG R: GGTTCAATACTCAATCGACTGAG	1264	17
aac(6')-Ie-*aph*(2'')-Ia	F: CCAAGAGCAATAAGGGCATA R: CACTATCATAACCACTACCG	220	18
ant(6)-Ia	F: ACTGGCTTAATCAATTTGGG R: GCCTTTCCGCCACCTCACCG	597	19
str	F: TATTGCTCTCGAGGGTTC R: CTTTCTATATCCATTCATCTC	646	8
ant(4')-Ia	F: GCAAGGACCGACAACATTTC R: TGGCACAGATGGTCATAACC	165	18
tet(K)	F: TTAGGTGAAGGGTTAGGTCC R: GCAAACTCATTCCAGAAGCA	697	20
tet(L)	F: CATTTGGTCTTATTGGATCG R: ATTACACTTCCGATTTCGG	456	

tet(M)	F: GTTAAATAGTGTTCTTGGAG R: CTAAGATATGGCTCTAACAA	576	
vanA	F: ATGGCAAGTCAGGTGAAGATGG R: TCCACCTCGCCAACAACTAACG	399	21
vanB	F: CAAAGCTCCGCAGCTTGCATG R: TGCATCCAAGCACCCGATATAC	484	22
mupA	F: CCCATGGCTTACCAGTTGA R: CCATGGAGCACTATCCGAA	419	23
fusB	F: CTATAATGATATTAATGAGATTTTTGG R: TTTTTACATATTGACCATCCGAATTGG	431	24
fusC	F: TTAAAGAAAAAGATATTGATATCTCGG R: TTTACAGAATCCTTTTACTTTATTTGG	332	
dfrA	F: CCTTGGCACTTACCAAATG R: CTGAAGATTCGACTTCCC	374	8
dfrD	F: TTCTTTAATTGTTGCGATGG R: TTAACGAATTCTCTCATATATATG	582	
dfrG	F: TCGGAAGAGCCTTACCTGACAGAA R: CCCTTTTTGGGCAAATACCTCATTCCA	323	16
dfrK	F: GAGAATCCCAGAGGATTGGG R: CAAGAAGCTTTTCGCTCATAAA	423	
Mutations and amino acid changes			
23S rRNA	F: GCGGTCGCCTCCTAAAAG	420	25

	R: ATCCCGGTCCTCTCGTACT		
rplC (L3)	F: ACCCTGATTTAGTTCCGTCTA	799	26
	R: GTT GACGCT TTAATGGGCTTA		
rplD (L4)	F: TCGCTTACCTCCTTAATG	1080	
	R: GGTGGAAACACTGTAACTG		
rplV (L22)	F: CAACACGAAGTCCGATTGGA	486	27
	R: GCAGACGACAAGAAAACAAG		
gyrA	F: ATGCGTGAATCATTCTTAGACTATGC	284	28
	R: GAGCCAAAGTTACCTTGACC		
grlA	F: TCGCAATGTATTCAAGTGGG	197	
	R: ATCGTTATCGATACTACCATT		
Virulence genes			
lukS/F-PV	F: ATCATTAGGTAAAATGTCTGGACATGATCCA	443	14
	R: GCATCAAGTGTATTGGATAGCAAAAGC		
tst	F: TTCACTATTTGTAAAAGTGTCAGACCCACT	180	29
	R: TACTAATGAATTTTTTTATCGTAAGCCCTT		
eta	F: ACTGTAGGAGCTAGTGCATTTGT	616	
	R: TGGATACTTTTGTCTATCTTTTTCATCAAC		
etb	F: CAGATAAAGAGCTTTATACACACATTAC	1553	
	R: AGTGAACTTATCTTTCTATTGAAAAACACTC		
etd	F: AACTATCATGTATCAAGG	402	30
	R: CAGAATTTCCCGACTCAG		
Immune Evasion Cluster (IEC)			
scn	F: AGCACAAGCTTGCCAACATCG	257	31

	R: TTAATATTTACTTTTTAGTGC	
chp	F: TTTACTTTTGAACCGTTTCCTAC	366
	R: CGTCCTGAATTCTTAGTATGCATATTCATTAG	
sak	F: AAGGCGATGACGCGAGTTAT	223
	R: GCGCTTGGATCTAATTCAAC	
sea	F: AGATCATTCGTGGTATAACG	344
	R: TTAACCGAAGGTTCTGTAGA	
sep	F: AATCATAACCAACCGAATCA	196
	R: TCATAATGGAAGTGCTATAA	

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