# Molecular basis of antimicrobial drug resistance in *Staphylococcus aureus* isolates recovered from young healthy carriers in Spain

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## ARTICLE INFO

## ABSTRACT

The frequency and genetic bases of antimicrobial drug resistance was determined for 111 *Staphylococcus aureus* recovered from young healthy carriers in a Spanish region. Resistances to ampicillin (84.7%), kanamycin (27%), erythromycin (25.2%), clindamycin (22.5%), tetracycline (11.7%), amikacin and tobramycin (6.3% each), gentamicin (5.4%), chloramphenicol (2.7%), ciprofloxacin (0.9%; MIC 4 μg/ml), moxifloxacin (0.9%) and mupirocin (0.9%; MIC 60 μg/ml) were found, and all were susceptible to methicillin (MSSA). Nearly 50% of the isolates were resistant to one antibiotic, 30% to two, 15.3% to three and 1.8% to four, while only 6.3% remained fully susceptible. A total of 31 profiles were found. For each phenotypic resistance, at least one gene accounting for it was identified. The detected genes were *blaZ*; *erm*(A)-*erm*(B)-*erm*(C)-*msr*(A)-*msr*(B)-*lnu*(A), *aphA-aadE-sat4-aacA* + *aphD-aadD*, *tet*(K), *cat*, and *qacA/B*, for resistance to ampicillin, macrolides and/or lincosamides, aminoglycosides, tetracycline, chloramphenicol, and quaternary ammonium compounds, respectively. In all isolates carrying *cat* genes, in all except one of the isolates positive for *tet*(K), and in most isolates with *blaZ*, *erm*(C), *msr*(A), or *msr*(B), the gene(s) mapped on resistance plasmids, which were detected in 69.2% of the resistant isolates (65% of the total). The *S. aureus* from young healthy carriers analysed in the present study do not constitute a reservoir of MRSA, but they represent a repository of multiple determinants conferring resistance to "old" antimicrobials. Some of these have still clinical applications and, considering the increasing resistance to recently introduced antimicrobials, none of them can be disregarded.

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## 1. Introduction

Along the last decades, the World Health Organization and the European Antimicrobial Resistance Surveillance System have increasingly recognized the importance of monitoring antimicrobial drug resistance in bacterial pathogens, and the genetic determinants involved, in order to provide the basis for prevention programs and assessment of their effectiveness. One of the major human pathogens is *Staphylococcus aureus*, a versatile bacteria capable of causing superficial and deep, often life threatening, infections [1], and which has gradually evolved towards resistance to all major classes of antimicrobial agents [2]. However, *S. aureus* is primarily a commensal organism which colonizes a high proportion of healthy humans, with estimations of 20—30% for persistent colonization [3]. The anterior nares are the main ecological niche of the bacterium. Other sites colonized include the throat, skin and gastrointestinal tract [4]. The carriage state has long been recognized as a risk factor for opportunistic infections, which occur at breached mucosal or cutaneous barriers [5,6]. It has been estimated that a large percentage of *S. aureus* infections in hospitals, including those affecting surgical wounds and causing bacteraemia, are of endogenous origin, and some studies have also identified carriage state as a risk factor for community-onset infections [5]. The relevance of *S. aureus* as a major human pathogen has prompted a wealth of studies on its epidemiology, virulence and resistance, both in hospital settings and the community, paying special attention to the methicillin resistant *S. aureus* (MRSA) clones [7]. The defining feature of the MRSA isolates is the staphylococcal cassette chromosome *mec* (SSC*mec*), which confers methicillin resistance. Apart from SCC*mec*, *S. aureus* contains other mobile genetic elements (MGEs) carrying diverse antimicrobial resistance genes [8—13]. In comparison with clinical isolates, studies on resistance in commensal *S. aureus* are comparatively scarce

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[14–17], and information on resistance plasmids, transposons and integrons is not available. The present study aimed to investigate the frequency, phenotypic patterns and genetic basis of antimicrobial drug resistance in *S. aureus* recovered in Spain from young healthy human carriers who have not association with health-care facilities.

## 2. Materials and methods

### 2.1. Bacterial isolates

A total of 111 isolates were included in this study. They have been collected at the Laboratory of Microbiology of the University of Oviedo (Spain), as part of training activities for biology students attending courses on Health Microbiology over several years (from 1997 to 2006). All individuals were properly informed of the study, and the sampling procedure does not represent a health risk. The students ranged from 18 to 22 years old, were healthy at the time of sampling, and did not had training courses at hospital facilities. These isolates, recovered from nasal swabs, have been previously characterized by means of SmaI-PFGE (pulsed field gel electrophoresis), *spa* typing, MLST (multilocus sequence typing), and for exotoxin gene content [18,19].

### 2.2. Antimicrobial susceptibility testing

All isolates were tested for antimicrobial susceptibility by the disk diffusion method, using commercially available discs (Oxoid, Madrid, Spain). The families and antimicrobials screened included beta-lactams [ampicillin (AMP), penicillin (PEN), oxacillin (OXA) and cefoxitin (FOX), both used as a substitute for methicillin], MLS group [erythromycin (ERY) and clindamycin (CLI)], aminoglycosides [gentamicin (GEN), tobramycin (TOB), kanamycin (KAN) and amikacin (AMK)], tetracycline (TET), chloramphenicol (CHL), trimethoprim−sulfamethoxazole (SXT), quinolones [ciprofloxacin (CIP) and moxifloxacin (MXF)], rifampicin (RIF), mupirocin (MUP), vancomycin (VAN), tigecycline (TGC), trimethoprim (TMP) and linezolid (LZD). Susceptibility to oleandomycin (OLE) was additionally tested in erythromycin susceptible strains with macrolide resistant genes. MICs (minimal inhibitory concentrations) for ciprofloxacin (Sigma−Aldrich, Spain), mupirocin (GlaxoSmithKline, Spain) and vancomycin (Laboratorios Normon SA, Madrid, Spain) were determined by the agar dilution method using concentrations ranging from 0 to 8 μg/ml, 0 to 256 μg/ml and 0 to 4 μg/ml, respectively. Results were scored according to the Clinical and Laboratory Standards Institute [20], except in the case of mupirocin resistance which was categorized as low (MIC 8−64 mg/l) and high (≥512 μg/ml) level resistance according to Patel et al. [21]. To estimate the rate of inducible lincosamide resistance, the double-disk diffusion test was performed on isolates that were susceptible to clindamycin and resistant to erythromycin, as reported [22]. *S. aureus* ATCC 29213, NCTC 8325 and several resistant isolates were included as controls [23].

### 2.3. Isolation of genomic DNA, PCR amplification and DNA sequencing of resistance determinants

DNA extraction and PCRs were performed as previously reported [23]. Primers for resistance genes and integrons (degenerate primers that detect *intI1*, *intI2* and *intI3* integrase genes) were previously described [23–25]. The genes tested conferred resistance to ampicillin−penicillin (*blaZ*), methicillin−oxacillin (*mecA*, *mecC* and SCC*mec* type), macrolides [*msr*(A), *msr*(B)], lincosamides [*lnu*(A) also named *lin*(A)], macrolides−lincosamides−streptogramins B [*erm*(A), *erm*(B), *erm*(C)], tetracyclines [*tet*(K), *tet*(L), *tet*(M), *tet*(O)], aminoglycosides (*aacA* + *aphD*, *aadD*, *aphA*, *aadE*, *sat4*), phenicols

(*cat*::pC194, *cat*::pC221, *cat*::pC223, *fexA*), mupirocin (*mupA*) and disinfectant compounds (*qacA/B*). The possible association of *blaZ* with Tn*552* was investigated by PCR amplification of a fragment spanning from the resistance gene to the transposase gene of this transposon [23]. Three primer pairs were used for detection of *cat* genes specifically associated with different plasmids (*cat*::pC194, *cat*::pC221 and *cat*::pC223) [23]. The mechanism of resistance to ciprofloxacin was addressed by sequencing DNA fragments of the quinolone-resistance determining region of the genes encoding the DNA gyrase (*gyrA* and *gyrB*) and the topoisomerase IV (*grlA* and *grlB*) genes, and of the promoter of the NorA transporter (*PnorA*) [26]. The nucleotide sequences were determined at SECUGEN S.L. (Madrid, Spain), and analysed with BlastN (http://www.ncbi.nlm.nih.gov) and ClustalW (http://www.ebi.ac.uk).

### 2.4. Plasmid analysis and Southern hybridization

Plasmid DNA was extracted by the alkaline lysis method with lysostaphin (Sigma−Aldrich) [27], and digested with HindIII following the manufacturer's instructions (Takara Bio Europe). The generated fragments were separated by electrophoresis on 0.75% w/v agarose gels in 0.5× TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA [pH 8]), transferred onto nylon membranes by Southern blotting [27], and hybridized with probes specific for the following resistance genes, previously reported in plasmids and found in the analysed isolates: *blaZ*, *p480* (Tn*552*), *erm*(C), *msr*(A), *msr*(B), *aacA* + *aphD*, *aadD*, *aphA*, *tet*(K), *cat*::pC194 or *cat*::pC221 [28–30]. The probes were generated with the PCR DIG labelling mix (Roche Diagnostics, Barcelona, Spain), which includes digoxigenin-labelled dUTP in addition to the four dNTPs. The amplicons were then purified with the GFX™ DNA and Gel Band Purification Kit (VWR, Barcelona, Spain), and the hybridizing fragments were detected with the "DIG Nucleic Acid Detection Kit" (Roche Diagnostics).

## 3. Results

### 3.1. Antimicrobial drug resistance in S. aureus isolates from young healthy carriers

All isolates were susceptible to oxacillin, cefoxitin, trimethoprim−sulfamethoxazole, rifampicin, vancomycin (MICs of 1 μg/ml), tigecycline, trimethoprim and linezolid. Only seven isolates were susceptible to all tested compounds (6.3%; S-profile), while the remaining isolates were grouped into 31 resistance profiles, labelled with R followed by a serial number (Table 1). The percentage of strains resistant to one antimicrobial was 48.7%, 30% for two, 15.3% for three, and 1.8% for four; the latter were considered as multidrug-resistant. Most resistance profiles (71%; 22/31) were represented by a single isolate; nine were shared by two or more isolates. The highest frequency of individual resistances corresponded to ampicillin−penicillin (84.7%), followed by kanamycin (27%), erythromycin (25.2%), clindamycin [22.5%; differentiated in clindamycin constitutive resistance together with macrolide resistance (8 isolates), clindamycin resistance inducible by macrolides (12 isolates) or clindamycin resistance without resistance to macrolides (5 isolates)], tetracycline (11.7%), amikacin, tobramycin (6.3%; each), gentamicin (5.4%), chloramphenicol (2.7%), ciprofloxacin-moxifloxacin and mupirocin (0.9%; each with one isolate). The MICs of the isolates resistant to ciprofloxacin and mupirocin were 4 μg/ml and 60 μg/ml, respectively.

### 3.2. Genetic basis of antimicrobial drug resistance

All isolates resistant to ampicillin−penicillin carried the *blaZ* β-lactamase gene, which was in most cases (78.7%; 74 of 94 *blaZ*

**Table 1**
Resistance profiles shown by *Staphylococcus aureus* recovered from healthy carriers and association with resistance plasmid profiles.

| R-profile[a] (N) | R-phenotype/R-genotype[b] | Resistance plasmid profile[c] (N) |
|---|---|---|
| S (7) | Susceptible | – (7) |
| R1a (7) | AMP/*blaZ* | – (4) |
| | AMP/**blaZ** | pR1, pR2 (2) |
| R1b (38) | AMP/*blaZ*::Tn*552* | – (14) |
| | AMP/**blaZ::Tn552** | pR3 (6), pR4 (10), pR5, pR6, pR7, pR10, pR11, pR12, pR13, pR15 |
| R1c | AMP-(OLE)/**blaZ::Tn552**-*msr*(B) | pR4 |
| R2 | ERY/[*msr*(A)-*msr*(B)]-*qacA/B* | – |
| R3a | [ERY-CLI[I]]/**blaZ::Tn552**-**erm(C)** | pR20 |
| R3b (2) | [ERY-CLI[I]]/**blaZ::Tn552**-[*msr*(B)-**erm(C)**] | pR4 |
| | [ERY-CLI[I]]/*blaZ*::Tn*552*-[**msr(B)**-**erm(C)**] | pR16 |
| R4 | CLI/*lnu*(A) | – |
| R5a | KAN/[*aphA*-*aadE*-*sat4*] | – |
| R5b | KAN/*blaZ*::Tn*552*-[*aphA*-*aadE*-*sat4*] | – |
| R6 | [AMK-KAN]/[*aphA*-*aadE*-*sat4*] | – |
| R7a (2) | AMP-ERY/*blaZ*-**msr(B)** | pR23 |
| | AMP-ERY/**blaZ**-**msr(B)** | pR22 |
| R7b | AMP-ERY/**blaZ::Tn552**-*msr*(B) | pR24 |
| R7c | AMP-ERY/**blaZ::Tn552**-[*msr*(A)-*msr*(B)] | pR9 |
| R8a | AMP-[ERY-CLI[I]]/*blaZ*-*erm*(C) | – |
| R8b (2) | AMP-[ERY-CLI[I]]/*blaZ*-[**msr(B)**-**erm(C)**] | pR16 |
| | AMP-[ERY-CLI[I]]/**blaZ**-[**msr(B)**-**erm(C)**] | pR19 |
| R8c | AMP-[ERY-CLI[I]]/*blaZ*::Tn*552*-**erm(C)** | pR17 |
| R8d | AMP-[ERY-CLI[I]]/**blaZ::Tn552**-[**msr(B)**-**erm(C)**] | pR21 |
| R8e | AMP-[ERY-CLI[I]]/**blaZ::Tn552**-[**msr(A)**-**msr(B)**-**erm(C)**] | pR25 |
| R9 | AMP-[ERY-CLI[C]]/**blaZ::Tn552**-[**msr(B)**-**erm(C)**] | pR18 |
| R10 | AMP-CLI/**blaZ::Tn552**-*lnu*(A) | pR3 |
| R11a | AMP-KAN/*blaZ*-[*aphA*-*aadE*-*sat4*] | – |
| R11b (7) | AMP-KAN/*blaZ*::Tn*552*-[*aphA*-*aadE*-*sat4*] | – (2) |
| | AMP-KAN/**blaZ::Tn552**-[*aphA*-*aadE*-*sat4*] | pR4, pR8, pR10 (2), pR12 |
| R11c | AMP-KAN/*blaZ*::Tn*552*-[*aphA*-*aadE*-*sat4*]-*qacA/B* | – |
| R12 | AMP-[AMK-KAN]/*blaZ*::Tn*552*-[*aphA*-*aadE*-*sat4*] | – |
| R13 | AMP-[AMK-GEN-KAN-TOB]/**blaZ::Tn552**-[*aacA* + *aphD*] | pR10 |
| R14a | AMP-TET/*blaZ*-**tet(K)** | pR32 |
| R14b (4) | AMP-TET/**blaZ::Tn552**-**tet(K)** | pR26 (2); pR28; pR36 |
| R15 | ERY-KAN/*msr(A)*-[*aphA*-*aadE*-*sat4*] | – |
| R16 | TET-[ERY-CLI[C]]/**blaZ::Tn552**-**erm(C)**-*tet*(K) | pR27 |
| R17 | AMP-[ERY-CLI[C]]-KAN/**blaZ::Tn552**-[**msr(B)**-**erm(C)**]-[*aphA*-*aadE*-*sat4*] | pR21 |
| R18 | AMP-[ERY-CLI[C]]-[GEN-KAN-TOB]/**blaZ::Tn552**-[**msr(B)**-**erm(C)**]-[*aacA* + *aphD*] | pR21 |
| R19 | AMP-[ERY-CLI[I]]-KAN/**blaZ::Tn552**-[*msr*(B)-*erm*(C)]-[*aphA*-*aadE*-*sat4*] | pR14 |
| R20 | AMP-[ERY-CLI[I]]-[AMK-GEN-KAN-TOB]/**blaZ::Tn552**-[**msr(B)**-**erm(C)**]-[**aphA**-*aadE*-*sat4*-**aacA** + **aphD**-*aadD*] | pR35 |
| R21a | AMP-[ERY-CLI[C]]-TET/*blaZ*::Tn*552*-[**msr(B)**-**erm(C)**]-**tet(K)** | pR30 |
| R21b | AMP-[ERY-CLI[C]]-TET/[*blaZ*::Tn*552*]-[**msr(B)**-*erm*(B)-**erm(C)**]-**tet(K)** | pR30 |
| R22 | AMP-[ERY-CLI[I]]-CHL/*blaZ*-*erm*(C)-**cat::pC194** | pR39 |
| R23 | AMP-[ERY-CLI[C]]-[CIP-MXF]/*blaZ*-[**msr(B)**-*erm*(B)-**erm(C)**]-[*glrA*(Ile45 to Met)-*glrB*(Asp422 to Glu)] | pR16 |
| R24a (2) | AMP-CLI-KAN/*blaZ*-*lnu*(A)-[*aphA*-*aadE*-*sat4*] | – |
| | AMP-CLI-KAN/**blaZ**-*lnu*(A)-[*aphA*-*aadE*-*sat4*] | pR1 |
| R24b | AMP-CLI-KAN/**blaZ::Tn552**-*lnu*(A)-[*aphA*-*aadE*-*sat4*] | pR5 |
| R25 (2) | AMP-KAN-TET/**blaZ::Tn552**-[*aphA*-*aadE*-*sat4*]-**tet(K)** | pR29; pR33 |
| R26 | AMP-[AMK-KAN-TOB]-TET/**blaZ::Tn552**-[**aphA**-*aadE*-*sat4*-*aadD*]-**tet(K)** | pR34 |
| R27 | AMP-[GEN-KAN-TOB]-TET/**blaZ::Tn552**-[*aacA* + *aphD*]-**tet(K)** | pR26 |
| R28 | AMP-[AMK-GEN-KAN-TOB]-TET/**blaZ::Tn552**-[**aphA**-*aadE*-*sat4*-**aacA** + **aphD**]-**tet(K)** | pR31 |
| R29 | AMP-KAN-CHL/**blaZ**-Tn*552*-[*aphA*-*aadE*-*sat4*]-**cat::pC194** | pR38 |
| R30 | AMP-ERY-KAN-CHL/**blaZ::Tn552**-*msr*(B)-[*aphA*-*aadE*-*sat4*]-**cat::pC221** | pR37 |
| R31 | AMP-[ERY-CLI[C]]-[AMK-GEN-KAN-TOB]-MUP/*blaZ*-[*msr*(A)-*msr*(B)-**erm(C)**-*lnu*(A)]-[**aphA**-*aadE*-*sat4*-*aacA* + *aphD*-*aadD*]-*qacA/B* | pR40 |

*N*, number of isolates when more than one. AMK, amikacin; AMP, ampicillin and penicillin; CHL, chloramphenicol; CIP, ciprofloxacin; CLI, clindamycin (C, constitutive; I, inducible); ERY, erythromycin; GEN, gentamicin; KAN, kanamycin; MXF, moxifloxacin; MUP, mupirocin; OLE, oleandomycin; TET, tetracycline; TOB, tobramycin. –, absence of plasmids or presence of plasmids which failed to hybridize with probes for resistance genes.

[a] Resistance (R) profiles are numbered according to R-phenotypes, and subtyped according to R-genotypes. Resistance to quaternary ammonium compounds was not tested.

[b] Resistance genes of plasmid location are shown in bold.

[c] Resistance plasmid profiles (pR) as shown in Table 2.

positive isolates) associated with transposon Tn*552* (Table 1). Five susceptible isolates carried anyhow the *blaZ* gene, alone (one isolate) or in combination with Tn*552* (4 isolates). Only three isolates were positive for the *qacA/B* genes, which are often located on β-lactamase/heavy metal resistance plasmids containing *blaZ* [28]. All isolates were negative for *mecA* and randomly selected isolates were also negative for *mecC*.

Resistance to erythromycin was detected either alone or together with clindamycin resistance (Table 1). Resistance to only erythromycin (8 isolates; profiles R2, R7, R15 and R30) was conferred by *msr*(A), *msr*(B) or *msr*(A) and *msr*(B) genes, present in one, five and two of isolates, respectively. One *msr*(B) positive isolate was erythromycin susceptible but resistant to oleandomycin (Table 1). Resistance to erythromycin together with inducible (12

isolates; profiles R3, R8, R19, R20 and R22) or constitutive (8 isolates; profiles R9, R16–R18, R21, R23 and R31) resistance to clindamycin, was always associated with *erm*(C). Some of these isolates carried this gene along with *msr*(B) (11 isolates), *msr*(A) and *msr*(B) (one isolate), *msr*(B) and *erm*(B) (two isolates) or *msr*(A), *msr*(B) and *lnu*(A) (one isolate). Five isolates were resistant to clindamycin but susceptible to erythromycin, and they were positive for the *lnu*(A) gene (profiles R4, R10 and R24).

Regarding to genes encoding aminoglycoside-modifying enzymes, *aacA + aphD* [also known as *aac(6′)-Ie + aph(2″)*] was found in 5.4% of the isolates (profiles R13, R18, R20, R27, R28 and R31), all resistant to gentamicin, kanamycin and tobramycin. The gene *aadD* [*ant(4′)-Ia*] was present in three isolates (2.7%), which were resistant to amikacin, kanamycin and tobramycin (profiles R20, R26 and R31). The gene *aphA* [*aph(3′)-IIIa*] was detected in 27 (24%) isolates resistant to kanamycin. All *aphA* positive isolates carried also the *aadE* [*ant(6)*] and *sat4* genes (which encode other aminoglycoside resistances: streptomycin and streptothricin, respectively), associated with transposon Tn*5405* [28–30].

With respect to other antimicrobials: i) all tetracycline resistant isolates contained the *tet*(K) gene; ii) the three chloramphenicol resistant isolates carried *cat* genes (encoding chloramphenicol acetyl transferases) associated with two types of previously reported plasmids (*cat*::pC194 or *cat*::pC221); iii) the moxifloxacin and ciprofloxacin (MIC 4 μg/ml) resistant isolate contained mutations in both grlA (Ile45-Met) and grlB (Asp422-Glu) genes; and iv) the mupirocin resistant isolate showed low level resistance (MIC 60 μg/ml) and was negative for the *mupA* gene which confers high level resistance (Table 1).

### 3.3. Involvement of potential mobile genetic elements in resistance

None of the isolates was positive for integrons, and extrachromosomal DNA could not be detected in susceptible isolates. In contrast, 82.7% of the resistant isolates carried plasmids, which were distributed into 53 HindIII-profiles. Forty of these, displayed by 69.2% of the resistant isolates, could be associated with resistance genes through hybridization experiments, and they were named pR followed by a serial number (Table 1, Fig. 1). Only eleven pRs were shared by more than one isolate.

Thirty four out of the 40 pRs included penicillinase plasmids, carrying *blaZ* or *blaZ*::Tn*552* together (18 profiles) or not (16 profiles) with other resistance genes (Table 1). In the remaining six pRs, the MLS resistance genes *msr*(B) and/or *erm*(C) (pR16, pR17 and pR23), the MLS genes *msr*(B) and *erm*(C) together with *tet*(K) (pR30), the *tet*(K) gene alone (pR32), and the *cat* gene of plasmid
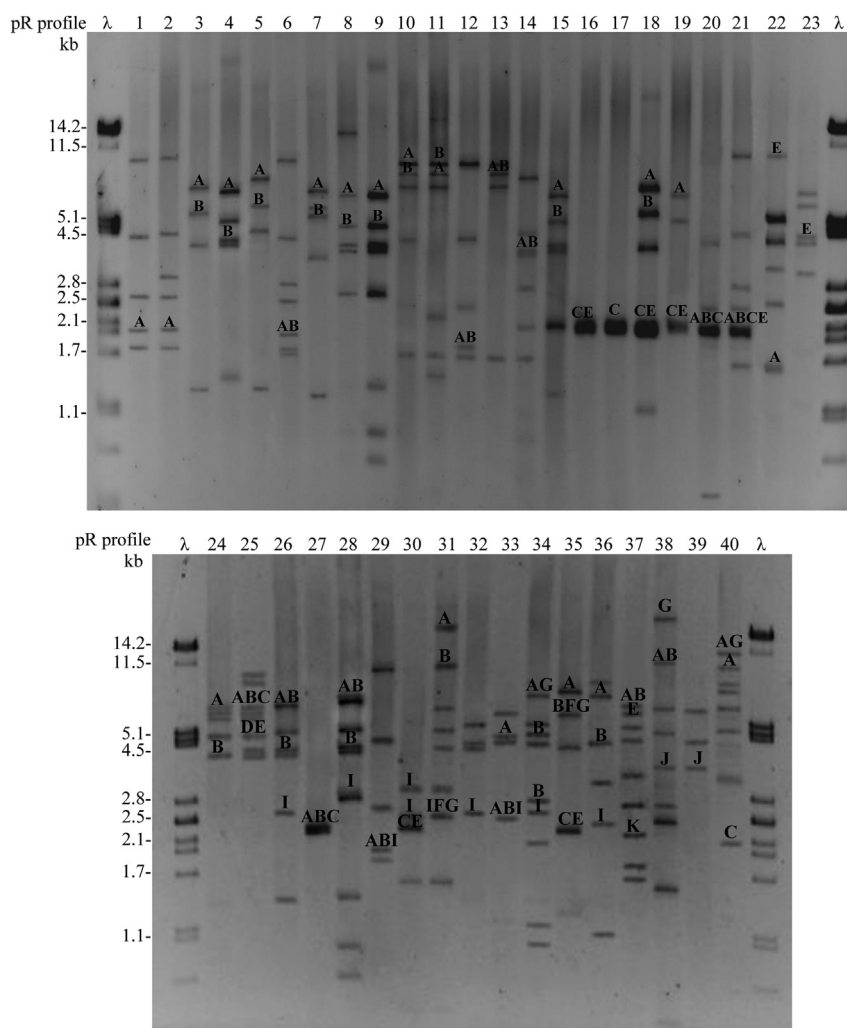


**Fig. 1.** HindIII pR plasmid profiles found in antimicrobial resistant *Staphylococcus aureus* from healthy carriers. Lane λ, lambda digested with PstI; lanes 1–40, profiles pR1–pR40. The probes that mapped on the different fragments are indicated as follows: A, *blaZ*; B, *p480*-Tn*552*; C, *erm*(C); D, *msr*(A); E, *msr*(B); F, *accA + aphD*; G, *aphA*; H, *aadD*; I, *tet*(K); J, *cat*:pC194; and K, *cat*:pC221.

pC194 (pR39) were mapped. Regarding to genes encoding aminoglycoside-modifying enzymes, none of the pR-profiles hybridized with the *aadD* probe, while in five isolates *aacA + aphD* and/or *aphA* mapped on plasmids (profiles pR31, pR34, pR35, pR38 and pR40). Each of the three isolates that carried *cat* genes showed a different pR: pR37 (*cat*::pC221), pR38 and pR39 (*cat*::pC194). In all, 100% of the *cat*, 67% of the *blaZ*, 92% of the *tet*(K), 80% of the *erm*(C), 65% of the *msr*(B), and 33% of the *aacA + aphD*, 20% of the *msr*(A), and 18.5% of *aphA* detected genes were plasmid located.

## 4. Discussion

Resistance studies on *S. aureus* have mainly been focused on isolates from patients and colonized staff in nosocomial settings, while information concerning the frequency of antimicrobial drug resistance and its genetic background in *S. aureus* recovered from healthy human carriers is relatively scarce [14–17]. In general, isolates from healthy carriers have lower frequencies of antimicrobial resistance than nosocomial isolates, except in the case of penicillin due to the widespread distribution of the *blaZ* gene among *S. aureus* [28,29]. In the healthy carrier isolates analysed in this study, resistances to gentamicin, tobramycin and ciprofloxacin, were lower than those reported for clinical isolates recovered in

Spanish hospitals during the 1986–2006 period (5.4% *vs.* 15%, 6.3% *vs.* 27% and 0.9% *vs.* 25.7%, respectively), while similar frequencies were obtained for erythromycin and clindamycin resistance (25.2% and 22.5% *vs.* 29.2% and 17%, respectively) [31]. However, this comparison has to be taken with caution since the study of Cuevas et al. [31] included a relatively high percentage of MRSA isolates, which are usually resistant to many other antimicrobials. In fact, the frequency of MRSA in Spanish hospitals ranged from 1.5% up to 29.2% during the 20 year period (average of 18%), while none of the 111 isolates from healthy carriers in Asturias, and only one out of 53 isolates (0.4%) recovered from healthy carriers in the nearby Spanish region of La Rioja [16], were MRSA. Similarly, no MRSA was detected in a recent study focused on the gut microbiota of healthy humans from Spain [32]. Regarding to other antimicrobials, resistances to chloramphenicol and mupirocin were low in both clinical and healthy carrier isolates (<9%); resistance to rifampicin and linezolid, which are still very rare in Spanish hospitals (0.6% and 0.2%, respectively), were not detected in healthy carrier isolates; and resistance to vancomycin and tigecycline were not recorded in any of the two settings [31].

The *S. aureus* isolates from young healthy carriers analysed in this study were highly heterogeneous with regard to resistance, with 15 genes detected combined into 31 resistance profiles. Most

**Table 2**
Resistance plasmid profiles shown *Staphylococcus aureus* recovered from healthy carriers and distribution among clonal complexes.

| pR profile[a] (N) | R-genotype | R-Profile (N) | Clonal complex[b] (N) |
|---|---|---|---|
| pR1 (2) | *blaZ* | R1a, R24a | CC5, CC30 |
| pR2 (2) | *blaZ* | R1a (2) | CC5 (2) |
| pR3 (7) | *blaZ*::Tn*552* | R1b (6), R10 | CC25 (3), CC45 (3), CC97 |
| pR4 (13) | *blaZ*::Tn*552* | R1b (10), R1c, R3b, R11b | CC15 (3), CC30 (5), CC45 (5) |
| pR5 (2) | *blaZ*::Tn*552* | R1b, R24b | CC15, CC25 |
| pR6 | *blaZ*::Tn*552* | R1b | CC5 |
| pR7 | *blaZ*::Tn*552* | R1b | CC59 |
| pR8 | *blaZ*::Tn*552* | R11b | CC30 |
| pR9 | *blaZ*::Tn*552* | R7c | CC15 |
| pR10 (4) | *blaZ*::Tn*552* | R1b, R11b (2), R13 | CC5 (2), CC25, CC30 |
| pR11 | *blaZ*::Tn*552* | R1b | CC5 |
| pR12 (2) | *blaZ*::Tn*552* | R1b, R11b | CC5, CC59 |
| pR13 | *blaZ*::Tn*552* | R1b | CC5 |
| pR14 | *blaZ*::Tn*552* | R19 | CC59 |
| pR15 | *blaZ*::Tn*552* | R1b | CC1 |
| pR16 (3) | *msr*(B), *erm*(C) | R3b, R8b, R23 | CC1, CC45, CC72 |
| pR17 | *erm*(C) | R8c | CC15 |
| pR18 | *blaZ*::Tn*552*, *msr*(B), *erm*(C) | R9 | CC25 |
| pR19 | *blaZ*, *msr*(B), *erm*(C) | R8b | CC1 |
| pR20 | *blaZ*::Tn*552*, *erm*(C) | R3a | CC5 |
| pR21 (3) | *blaZ*::Tn*552*, *msr*(B), *erm*(C) | R8d, R17, R18 | CC5 (3) |
| pR22 | *blaZ*, *msr*(B) | R7a | CC15 |
| pR23 | *msr*(B) | R7a | CC45 |
| pR24 | *blaZ*::Tn*552* | R7b | CC5 |
| pR25 | *blaZ*::Tn*552*, *msr*(A), *msr*(B), *erm*(C) | R8e | CC45 |
| pR26 (3) | *blaZ*::Tn*552*, *tet*(K) | R14b, R27 | CC15 (2), CC30 |
| pR27 | *blaZ*::Tn*552*, *erm*(C) | R16 | CC30 |
| pR28 | *blaZ*::Tn*552*, *tet*(K) | R14b | CC15 |
| pR29 | *blaZ*::Tn*552*, *tet*(K) | R25 | CC97 |
| pR30 (2) | *msr*(B), *erm*(C), *tet*(K) | R21a, R21b | CC45 (2) |
| pR31 | *blaZ*::Tn*552*, *tet*(K), *accA + aphD*, *aphA* | R28 | CC30 |
| pR32 | *tet*(K) | R14a | CC8 |
| pR33 | *blaZ*::Tn*552*, *tet*(K) | R25 | CC30 |
| pR34 | *blaZ*::Tn*552*, *tet*(K), *aphA* | R26 | CC1 |
| pR35 | *blaZ*::Tn*552*, *msr*(B), *erm*(C), *accA + aphD*, *aphA* | R20 | CC25 |
| pR36 | *blaZ*::Tn*552*, *tet*(K) | R14b | CC45 |
| pR37 | *blaZ*::Tn*552*, *msr*(B), *cat*::pC221 | R30 | CC15 |
| pR38 | *blaZ*::Tn*552*, *aphA*, *cat*::pC194 | R29 | CC97 |
| pR39 | *cat*::pC194 | R22 | CC9 |
| pR40 | *blaZ*, *erm*(C), *aphA* | R31 | CC25 |

*N*, number of isolates when more than one.
[a] Resistance plasmid profiles (pR) were obtained by digestion of extracted plasmid DNA with HindIII, and subsequent Southern blot hybridization with specific probes for the following resistance genes: *blaZ*, *p480* (Tn*552*), *msr*(A), *msr*(B), *erm*(C), *aphA*, *accA + aphD*, *aadD*, *tet*(K), *cat*::pC194, and *cat*::pC221.
[b] Clonal complexes (CCs) have been previously identified in Ref. [19].

of these genes (*erm*(C), *tet*(K), *aacA* + *aphD*, *aadD* and *aphA*) have also been found in healthy carrier isolates from La Rioja, Germany or Switzerland [14,16,17,32]. However, the *erm*(A) gene, which was not found in healthy carrier isolates from Asturias, was reported at frequencies ranging from 1.9 to 11% in these other studies and, at a low frequency, in foodborne isolates from Asturias [23]. Interestingly, all macrolide—lincosamide resistant foodborne isolates [including those carrying *erm*(A)] were also positive for *erm*(C), which appears to be the most frequent macrolide—lincosamide resistance determinant in the *S. aureus* circulating in our region.

In the present study, at least one gene accounting for each phenotypic resistance was found, and the presence of more than one gene conferring resistance to the same antimicrobial, or to antimicrobials belonging to the same family, was common. As a relevant example, five (*aphA*, *aadE*, *sat4*, *aacA* + *aphD* and *aadD*) and four [*erm*(C), *msr*(A), *msr*(B) and *lnu*(A)] genes conferring resistance to aminoglycosides and MLS, respectively, were detected in a single multidrug resistant isolate (R31 profile). This will contribute to maintenance of the phenotypic resistance, even in the absence of selective pressure.

Gathering in a single isolate of multiple resistant determinants, active against the same or different classes of antimicrobials, is commonly mediated by acquisition of mobile genetic elements, such as transposons and plasmids [28—30], and this seems to be the case in the present study. For instance, in most of the isolates resistant to penicillin, the *blaZ* gene was associated with Tn*552* and the simultaneous presence of *aadE* and *sat4* in all *aphA*-positive isolates strongly support the location of the three genes within Tn*5405* [33]. In addition, plasmids were detected in 82.7% (86 of 104 isolates) of the resistant isolates, a frequency which fits within the range reported for MRSA isolates (60% up to 100%) [8,9,13]. For 69.2% (72 of 104 isolates) of the resistant isolates, an association between plasmid and resistance could be established by means of hybridization experiments. In fact i) *blaZ* and *blaZ*::Tn*552* were located on plasmids in most positive isolates; ii) in all except one of the tetracycline resistant isolates the *tet*(K) efflux gene was plasmid-borne; iii) in all chloramphenicol resistant isolates, the *cat* genes were also carried by plasmids; and iv) in most isolates positive for *erm*(C) and *msr*(B) the genes mapped on plasmids. Accordingly, extrachromosomal DNA appears to be playing a relevant role in maintenance and spread of antimicrobial resistance in commensal *S. aureus*.

A large number of *S. aureus* plasmids have recently been sequenced and assigned to 39 groups, each with a unique combination of *rep* genes [34]. An association of resistance and virulence genes with plasmid groups, and of plasmids groups with *S. aureus* lineages, could be established. The healthy carrier isolates from Asturias were previously assigned to twelve clonal complexes [19]: CC30 (27%), CC5 (18.9%), CC45 (16.2%), CC15 (11.7%), CC25 (8.1%), CC1, CC9 (3.6% each), CC59, CC97 and CC121 (2.7% each), CC72 (1.8%) and CC8 (0.9%). In the present study (see Table 2), the *blaZ* gene was associated with 35 pR profiles distributed among eight out of the twelve CCs (CC1, CC5, CC15, CC25, CC30, CC45, CC59 and CC97); MLS genes [*erm*(C), *msr*(A) and *msr*(B)] with 14 pRs in seven CCs (CC1, CC5, CC15, CC25, CC30 CC45 and CC72); aminoglycoside resistance genes (*aacA* + *aphD* and/or *aphA*) with five pRs in four CCs (CC1, CC25, CC30 and CC97); the *tet*(K) probe with nine pRs in six CCs (CC1, CC8, CC15, CC30, CC45, CC97); and the plasmid-*cat* genes with three pRs in three CCs (CC9, CC15 and CC97). It is of note that CC121 was the only lineage in which resistance plasmids were not detected. However, for a better understanding of the distribution of resistance plasmids among these clonal complexes, a deeper study based on *rep* typing and sequencing has to be performed.

In summary, the *S. aureus* from young healthy carriers analysed in the present study do not constitute a reservoir of MRSA, but they represent a repository of multiple determinants conferring resistance to "old" antimicrobials. Some of these have still clinical applications and, considering the increasing resistance to recently introduced antimicrobials, none of them can be disregarded. Such isolates could be eventually involved in disease and/or act as recipients of SCC*mec*, stressing the interest of the pool of commensal *S. aureus* circulating in the community.

## Acknowledgements

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