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Genetic environment and location of the *lnu*(A) and *lnu*(B) genes in methicillin-resistant *Staphylococcus aureus* and other staphylococci of animal and human origin

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Objectives: To detect the presence of *lnu* genes in staphylococcal strains with the unusual phenotype lincosamide resistance/macrolide susceptibility (L^R/M^S), and to determine their locations and genetic environments.

Methods: Six staphylococcal strains of human and animal origin with the phenotype L^R/M^S were studied. The presence of 15 resistance genes was tested by PCR. SCC*mec* typing was performed for all methicillin-resistant strains. *agr* typing, *spa* typing and multilocus sequence typing were carried out for *Staphylococcus aureus* strains. Transformation experiments were carried out by electrotransformation. Plasmid or chromosomal gene location was determined by Southern blot analysis and the genetic environments of the *lnu* genes were studied in all strains.

Results: Three methicillin-resistant staphylococcal strains contained the *lnu*(A) gene. The presence of the pLNU1 plasmid carrying *lnu*(A) was confirmed in one methicillin-resistant *S. aureus* (MRSA) ST398-t108 and one methicillin-resistant *Staphylococcus sciuri*. A novel *lnu*(A)-carrying plasmid (pUR5425) was identified in one MRSA ST125-t067 strain. Transformants of the three *lnu*(A)-positive strains presented increased lincomycin MIC values. The remaining three studied staphylococcal strains harboured the *lnu*(B) gene: two methicillin-susceptible *S. aureus* (MSSA) ST9-t337 and one MRSA ST398-t011. The *lnu*(B) gene was embedded in the chromosome in the two MSSA strains and in a large-sized plasmid in the MRSA strain. The same *lnu*(B) genetic environment was detected in these three strains.

Conclusions: The resistance phenotype L^R/M^S seems to be related to *S. aureus* animal-associated clonal lineages (ST398 and ST9). A novel *lnu*(A)-carrying plasmid was identified and this is the first detection of the *lnu*(B) gene in MRSA ST398.

Keywords: ST398, ST9, pLNU1, lincosamide resistance, *S. aureus*, MRSA

Introduction

The lincosamides constitute a group of antibiotics employed to treat staphylococcal and streptococcal infections in human and veterinary medicine. This group is classified together with macrolides and streptogramins (group MLS), owing to the fact that they share their binding site. The main mechanism of resistance to these MLS antimicrobials in staphylococci is encoded by *erm* genes.[1] In contrast, *lnu* genes confer resistance only to lincosamides. Five *lnu* genes have been described so far: *lnu*(A), *lnu*(B), *lnu*(C), *lnu*(D) and *lnu*(F). A priori, these genes only confer resistance to lincomycin and pirlimycin, although resistance to clindamycin has been also suggested.[2] Recently, the detection of the unusual resistance phenotype lincosamide resistance/macrolide susceptibility (L^R/M^S) has increased among staphylococcal strains of animal origin.[3,4]

The aims of this study were to determine the presence of *lnu* genes, their locations and genetic environments in methicillin-resistant *Staphylococcus aureus* (MRSA) and in other staphylococcal strains that showed the unusual resistance phenotype L^R/M^S.

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Materials and methods

Bacterial strains

Six *Staphylococcus* spp. strains, which showed the unusual resistance phenotype L^R/M^S, were included in this study: five *S. aureus* [three MRSA and two methicillin-susceptible *S. aureus* (MSSA)] and one methicillin-resistant *Staphylococcus sciuri* (MRSC).

Antimicrobial susceptibility testing

Susceptibility testing was performed by the disc diffusion method following CLSI guidelines.[5] The tested antibiotics were as follows: penicillin, oxacillin, cefoxitin, erythromycin, clindamycin, gentamicin, streptomycin, kanamycin, tobramycin, tetracycline, ciprofloxacin, chloramphenicol, trimethoprim/sulfamethoxazole, vancomycin, teicoplanin, mupirocin and fusidic acid. In addition, the MICs of clindamycin, lincomycin, erythromycin, linezolid, tiamulin, virginiamycin and chloramphenicol were determined by the agar dilution method.[5,6] CLSI breakpoints were used for all antibiotics,[5] except for lincomycin, for which Société Française de Microbiologie breakpoints were considered,[6] and for tiamulin and virginiamycin due to the lack of officially approved breakpoints.

Resistance genotype

The presence of the *lnu*(A), *lnu*(B), *lnu*(C) and *lnu*(D) genes was analysed by PCR and sequencing (Table S1, available as Supplementary data at *JAC* Online). The presence of the *vga*(A), *vga*(B), *vga*(C), *vga*(E), *lsa*(B), *cfr*, *tet*(K), *tet*(L), *tet*(M), *tet*(O), *aacA-aphD*, *aadD*, *aphA3*, *dfrS1*, *dfrD*, *dfrG* and *dfrK* resistance genes was also studied by PCR, as previously described.[4,7]

Molecular typing

Two of the strains (C1841 and C2944) were typed in previous studies.[7,8] The remaining *S. aureus* strains were typed [*spa* typing, *agr* typing and multilocus sequence typing (MLST)] as previously described.[7] SCC*mec* typing was performed for the methicillin-resistant strains.[7] Moreover, due to the similar genetic background of the two MSSA strains, PFGE was performed as recommended.[9]

Location of the lnu genes

A plasmid or chromosomal gene location was determined by Southern blot analysis. Two methods were used to detect a possible plasmid location: the S1-PFGE assay and plasmid DNA extraction.[4,10] Moreover, I-CeuI-PFGE was performed to detect a possible chromosomal location. Hybridizations were carried out with *lnu*(A) and *lnu*(B) probes, and a 16S rDNA probe (in the case of I-CeuI), according to the manufacturer's recommendations (Roche).

Transformation experiments and genetic environment of the lnu(A) gene

Transformation experiments were performed by electrotransformation into the recipient strain *S. aureus* RN4220.[10] Putative transformants were selected on brain heart infusion plates containing lincomycin (8 mg/L). The genetic environments of the *lnu*(A) genes were studied by inverse PCR and sequencing. The primers used for amplification were *lnu*(A)inv-F and *lnu*(A)inv-R (Table S1, available as Supplementary data at *JAC* Online). Plasmids containing the *lnu*(A) gene were completely sequenced by primer walking using the PCR primers as the initial primers.

Genetic environment of the lnu(B) gene

To determine the genetic environment of the *lnu*(B) gene, primers were designed based on the previously reported pEF418 structure (GenBank accession number AF408195.1) (Table S1, available as Supplementary data at *JAC* Online). In addition, whole-cell DNA of the MRSA C2944 strain was digested with PvuII (New England Biolabs) and subjected to ligation with T4 ligase (Fermentas). An inverse PCR was performed using the 1_inv-F and 2_inv-R primers (Table S1, available as Supplementary data at *JAC* Online and see Figure 1a), and the amplicon was sequenced. Due to the identification of IS*257* upstream of the detected genetic structure, primers were designed to determine whether the same insertion sequence was also downstream of the *lnu*(B) gene (Table S1, available as Supplementary data at *JAC* Online). Primers based on the structure detected in the C2944 strain were designed and tested in C2828 and C2829. Putative circular forms were also investigated by inverse PCR and sequencing using the primers 2_inv-R and pEF418-4-F (Table S1, available as Supplementary data at *JAC* Online).

Results and discussion

Two MRSA strains (ST398 and ST125) of animal and human origin, respectively, and one MRSC of animal origin carried the *lnu*(A) resistance gene. Moreover, three strains (one MRSA ST398 and two MSSA ST9) of human origin revealed the presence of the *lnu*(B) gene (Table 1). Both MSSA strains showed closely related patterns by PFGE. Previous reports indicated that the MRSA lineage ST398 is a livestock-associated (LA) MRSA, frequently encountered in farm animals, although it is appearing in the human population.[11] Another identified LA staphylococcal lineage is ST9, which has been reported in animal MRSA and MSSA isolates.[12,13] It seems that there is a relationship between the *lnu*(A) and *lnu*(B) resistance genes and animal clonal lineages of *S. aureus*. This fact has been suggested before, since other resistance genes responsible for the unusual resistance phenotype L^R/M^S have been detected in MRSA ST398 strains.[3,4,14] The use of lincosamides in veterinary medicine could explain the presence of these resistance genes in strains of animal origin.[4] In this study, the *lnu*(A) gene was also identified in an MRSA ST125 strain of human origin. The ST125 lineage is the most commonly found hospital-acquired MRSA clone in Spain and the detection of this gene in a human strain is remarkable.[1,15]

The MIC values obtained for the *lnu*(A)-positive strains are in concordance with the results obtained by others.[10] However, MRSC strain C2376 showed a higher tiamulin MIC value than the remaining *lnu*(A)-positive strains (Table S2, available as Supplementary data at *JAC* Online). None of the tested genes involved in tiamulin resistance was detected in strain C2376. Only the MIC value of lincomycin was increased for transformant strains with respect to the recipient strain (Table S2).

The three *lnu*(B)-positive strains presented higher MIC values of lincomycin, clindamycin and tiamulin than the *lnu*(A)-positive strains (Table S2). The Lnu(A) protein modifies a hydroxyl group of clindamycin and lincomycin at positions 3 and 4, respectively. Nevertheless, the Lnu(B) protein modifies a hydroxyl group at position 3 in both antimicrobials.[2] This fact and the presence of a gene encoding an ABC transporter upstream of the *lnu*(B) gene in our strains (Figure 1a) could explain the detected phenotype. Interestingly, this ABC transporter showed a similarity of 76.5% with respect to the staphylococcal Lsa(B) protein.[16]

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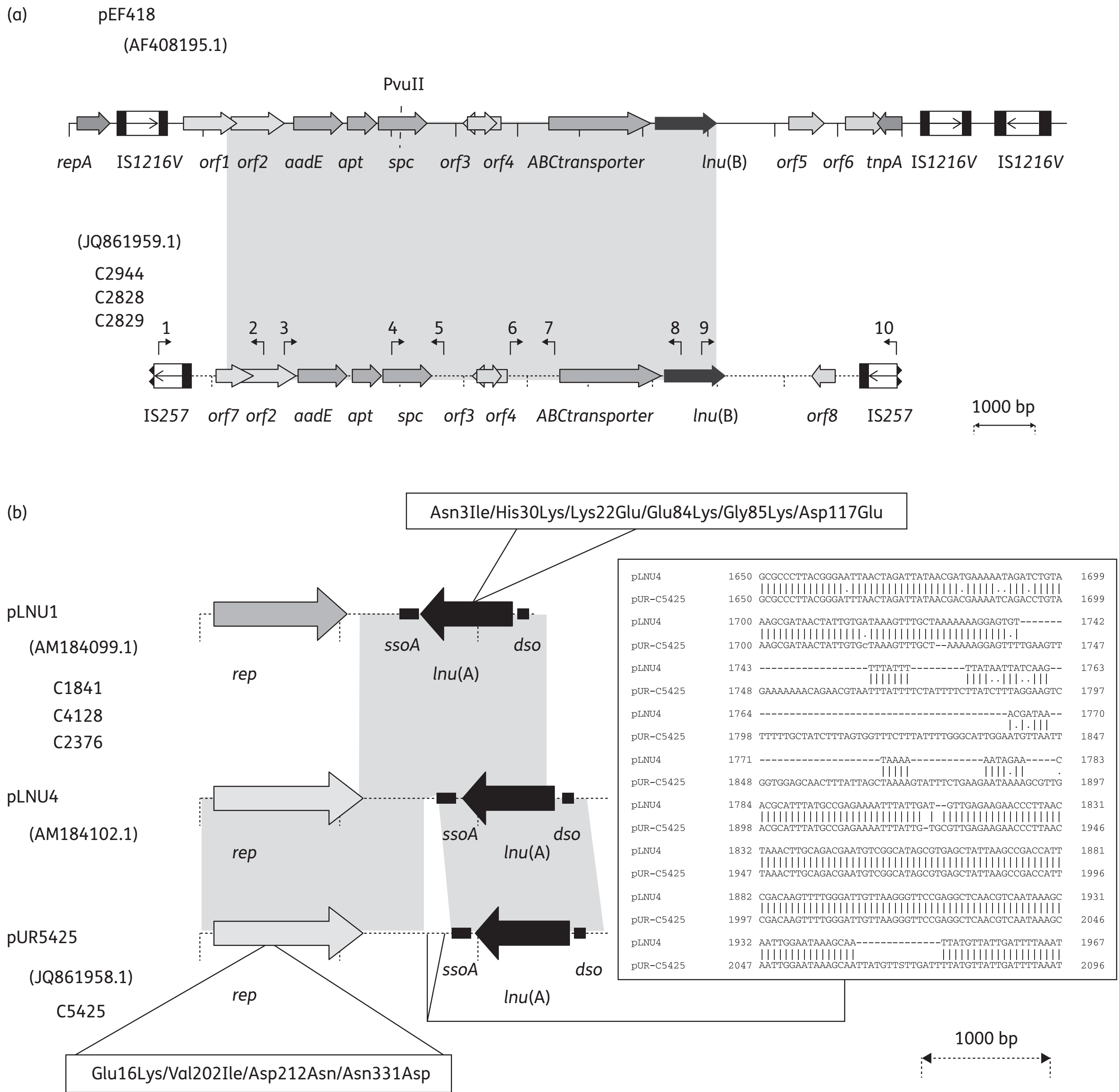

Figure 1. (a) Structure of the novel genetic environment of the *lnu*(B) gene (strain C2944) and the structure of the already described *Enterococcus* plasmid pEF418. The PvuII cleavage site is shown. The positions of primers used to amplify the genetic environment of the *lnu*(B) gene are indicated by arrows and numbers as follows: 1, C2944-tnp-F; 2, 2_inv-R; 3, 1_inv-F; 4, pEF418-2-F; 5, pEF418-3-R; 6, pEF418-1-F; 7, pEF418-2-R; 8, pEF418-1-R; 9, pEF418-4-F; and 10, IS*257*-R. (b) Structure of the novel plasmid containing the *lnu*(A) gene (strain C5425) and the already described plasmids pLNU1 and pLNU4. Some of the differences between pLNU4 and the novel plasmid pUR5425 are shown in boxes. Amino acid changes in the lincosamide nucleotidyltransferase protein between pLNU1 and pUR5425 (with a lincosamide nucleotidyltransferase similar to pLNU4) are also shown in a box.

Hybridization experiments revealed the location of the *lnu*(A) gene in small plasmids (from 2.3 to 2.7 kb) in our strains. Sequencing of the inverse PCR products showed that the MRSA ST398 strain and the MRSC strain harboured a *lnu*(A) plasmid identical to pLNU1 (with a GC content of 29% and a size of 2361 bp) of *Staphylococcus chromogenes* (GenBank AM184099.1).

The MRSA ST125 strain presented a novel *lnu*(A)-plasmid (named pUR5425) of 2690 bp, 31% GC content and with a

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Table 1. Characteristics of the *Staphylococcus* spp. strains included in this study

Strain	MRSA/ MSSA/MRSC	Date of isolation	Sample/origin	MLST/*spa* type	SCC*mec* type	*agr* type	Resistance phenotype[a]	Resistance genotype	Location of *lnu* genes	GenBank number
C1841	MRSA	January 2009	nasal/pig	ST398/ t108	V	I	PEN-OXA-FOX-CLI^{I}-LIN-TET-SXT	***lnu*(A)**, *mecA*, *tet*(L), *dfrK*	pLNU1 plasmid (2.3 kb)	AM184099.1
C5425	MRSA	August 2009	unknown/ human	ST125/ t067	IV	II	PEN-OXA-FOX-LIN-CIP	***lnu*(A)**, *mecA*	pUR5425 plasmid (2.7 kb)	JQ861958.1 new
C2376	MRSC	August 2009	nasal/pig	—	III	—	PEN-OXA-FOX-CLI^{I}-LIN-TET-FUS^{I}	***lnu*(A)**, *mecA*, *tet*(L)	pLNU1 plasmid (2.3 kb)	AM184099.1
C2828	MSSA	December 2009	skin lesion/ human	ST9/t337	—	II	PEN-CLI-LIN-TET-STR-GEN-KAN-TOB-CIP	***lnu*(B)**, *tet*(K), *tet*(L), *aacA-aphD*, *aadD*	chromosome	JQ861959.1 new
C2829	MSSA	January 2010	skin lesion/ human	ST9/t337	—	II	PEN-CLI-LIN-TET-STR-GEN-KAN-TOB-CIP	***lnu*(B)**, *tet*(L), *aacA-aphD*, *aadD*	chromosome	JQ861959.1 new
C2944	MRSA	January 2009	ear swab lesion/human	ST398/ t011	V	I	PEN-OXA-FOX-CLI-LIN-TET-STR-GEN-KAN-TOB-CIP	***lnu*(B)**, *mecA*, *tet*(M), *aacA-aphD*	plasmid (250 kb)	JQ861959.1 new

PEN, penicillin; OXA, oxacillin; FOX, cefoxitin; CLI, clindamycin; LIN, lincomycin; TET, tetracycline; STR, streptomycin; GEN, gentamicin; KAN, kanamycin; TOB, tobramycin; FUS, fusidic acid; SXT, trimethoprim/sulfamethoxazole; CIP, ciprofloxacin; ^{I}, intermediate.

[a]CLSI breakpoints and methodology were used for all antibiotics, except for lincomycin, for which Comité de l'Antibiogramme de la Société Française de Microbiologie breakpoints and methodology were considered.

high similarity (92.8%) to the previously described plasmid pLNU4 (GenBank AM184102.1). This novel plasmid was registered in GenBank (JQ861958.1). The most notable differences between pUR5425 and pLNU4 were the presence of amino acid changes in the Rep protein, and several variations in the region between the *rep* gene and the single-strand origin (Figure 1b). The Lnu(A) protein encoded by the plasmids detected in this study presented some amino acid exchanges. Variability of the Lnu(A) amino acid sequence has already been described.[10]

No transformants were obtained from *lnu*(B)-positive strains. Few data exist on the location of the *lnu*(B) gene and its genetic environment. A plasmid location (~240 kb) was suggested in the first description of this gene.[17] Additionally, in that study, it was observed that the *lnu*(B) gene could be integrated into the chromosome of the obtained transconjugant. These results are in agreement with our observation since this gene was embedded in the chromosome of our two MSSA strains and in a large-sized plasmid (250 kb) in our MRSA strain C2944. Up to now, only a partial sequence of one *lnu*(B)-carrying plasmid (pEF418) of *Enterococcus faecalis* is available in GenBank. In our strains, the region that immediately preceded the *lnu*(B) gene was homologous to pEF418 and this region included other resistance genes, such as *aadE* or *spc* (Figure 1a). Moreover, two insertion sequences (both IS*257*) flanked the structure detected and a circular structure was identified (Figure 1a). This fact indicates that the lateral transfer could occur possibly mediated by the presence of the two insertion sequences that would enable the mobilization of the *lnu*(B) gene. Further studies are required to confirm this hypothesis. The genetic environment of the *lnu*(B) gene detected in strain C2944 was also identified in the two other *lnu*(B)-positive strains studied and was registered in GenBank (JQ861959.1).

The transference of resistance genes among bacterial genera and species has been corroborated in this study. Firstly, two of the *lnu*(A)-positive strains (one MRSA and one MRSC) contained this gene in the plasmid pLNU1, which was previously described in *S. chromogenes*.[10] The transference of this gene among different staphylococcal species is expected since the *lnu*(A) gene has been detected in *S. aureus*, *Staphylococcus pseudintermedius*, *S. chromogenes*, *Staphylococcus simulans*, *Staphylococcus haemolyticus* and *Staphylococcus epidermidis*.[8,10,18] Moreover, the *lnu*(B) gene was identified in three *S. aureus* strains in this study. This gene has been detected in *Enterococcus* spp., *Streptococcus* spp. and *Clostridium* spp.[17,19,20] Regarding staphylococci, there is no information in the National Center for Biotechnology Information (NCBI) database about strains harbouring the *lnu*(B) gene. However, according to the web site http://faculty.washington.edu/marilynr/ermweb4.pdf, this gene has been already detected in the *Staphylococcus* genus. Therefore, our study describes the first report of this gene in MRSA of the lineage ST398.

In conclusion, the unusual resistance phenotype L^R/M^S seems to be related to animal clonal lineages of *S. aureus*. We describe a novel *lnu*(A)-carrying plasmid and, to the best of our knowledge, this is the first description of the *lnu*(B) gene in MRSA ST398.

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Part of these data were previously presented at an international conference (Abstract 27, Second ASM-ESCMID Conference on

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Methicillin-resistant Staphylococci in Animals: Veterinary and Public Health Implications, Washington, DC, 2011) and at a national congress (Abstract 695, Fifteenth Congress of the Spanish Society of Infectious Diseases and Clinical Microbiology, Malaga, Spain, 2011).

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Transparency declarations
None to declare.

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

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