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Resistance Genes Underlying the LS_A Phenotype of Staphylococcal Isolates from France

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There exist numerous genes disseminated by mobile elements that can confer cross-resistance to lincosamides and streptogramin A compounds in staphylococci. This study investigated the nature and means of dissemination of genes responsible for LS_A resistance among 24 French clinical isolates screened for reduced susceptibility to lincomycin. The *vga*(A)*v* gene was found to be the most prevalent determinant of LS_A resistance, while Tn*5406* appeared to be its exclusive gene support.

Combined resistance to lincosamides and streptogramin A (S_A) compounds is referred to as the LS_A phenotype in clinical microbiology laboratories. The resistance phenotype relies on the presence of the ARE subfamily of class 2 ATP-binding cassette (ABC) ATPases, a class of ABC proteins made up of two homologous ABC ATPase domains separated by a flexible linker without any identifiable transmembrane domains. Despite the efforts of several laboratories, the resistance mechanism remains elusive. Two hypotheses have been proposed: protection of the ribosome by preventing access to the antibiotic binding site or antibiotic efflux (1). Direct evidence supporting the first hypothesis is still lacking, while many experimental data have recently sustained the second one. Reports on membrane localization of ARE proteins Vga(A) and VmlR, formerly ExpZ (2), in bacteria showing either acquired or intrinsic resistance to LS_A antibiotics (3, 4), along with transport assays using the radiolabeled lincomycin compound (5), have given credit to the hijacking hypothesis, wherein the ABC protein alters the specificity of at least one transporter (1). The flexible linker between each ATPase domain is presumed to be the drug-binding region of the ARE proteins (5, 6).

Contrary to Cfr- or Erm-based methylations of 23S rRNA that confer resistance to numerous translation inhibitors (7, 8), the ABC-mediated resistance mechanism is limited to only a few antimicrobials, macrolides and streptogramin B (S_B) compounds or lincosamides and S_A compounds, depending on what kind of ARE protein is present in the bacterial strain (see http://faculty.washington.edu/marilynr/ for any detailed information). Due to their drug specificity, ARE proteins can be divided into two separate clusters: those in the first cluster confer various degrees of resistance to lincosamides, S_A compounds, and pleuromutilins, while those in the second cluster confer various degrees of resistance to macrolides and S_B compounds. Within each cluster, the linker regions of the ARE proteins may be strongly dissimilar, pointing to their possible involvement, outside the drug resistance spectrum, in certain interactions with membrane partners (4, 9). Mapping of the diversity within ARE proteins is thus helpful for deciphering the resistance mechanism but also for epidemiological surveys.

The goal of the present study was to analyze the gene diversity occurring among various staphylococcal species with respect to the LS_A phenotype. Here we also report the manner in which the

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TABLE 1 Oligodeoxyribonucleotides used in PCR experiments for diagnosis and cloning purposes[a]

Primer	Nucleotide sequence (5′ to 3′)	DNA target	Program[b]	Amplicon size
V1	AAGTGGTGGTGAAGTAACACG	*vga*(A)	P1 diagnosis	1 kbp
V2	TCAAGAAAGTTTGTTGGTTCATC			
T1	ATATCCGACATCGCCAACTC	Tn*5406*	P1 diagnosis	740 bp
T2	TTCGTGTTACTTCACCACCAC			
X	ATTTCATTATCGCCATCTGTC	EES57021	P2 cloning	2.4 kbp
Y	TCTTCCTTCTTCAATTTCCC			
lnuACDf	GGTTWGATGGWGGYTGGGG	*lnu*(A)	P3 diagnosis	230 bp
lnuAr	AATTGCCACCTTCTGGGTTTGC			
lnuBFf	AGARGGTGACSARTWCTCTGA	*lnu*(B)	P3 diagnosis	400 bp
lnuBFr	AKGMRCGAGCATAYTCTCC			

[a] The primer pairs described here were not found in the literature. Other primer pairs were vgaB1 and vgaB2 for detection of *vga*(B) (24), vgaC-1 and vgaC-2 for detection of *vga*(C) (21), vgaE_fw and vgaE_rv for detection of *vga*(E) (22), lsaB-1 and lsaB-2 for detection of *lsa*(B) (21), cfr_fw and cfr_rv for detection of *cfr* (23), vatA1 and vatA2 for detection of *vat*(A) (24), and 16S-27F and 16S-907R for detection of *rrs* (12).

[b] Thermocycling programs were as follows: P1, 1 cycle of 5 min at 94°C and 2 min at 54°C; 30 cycles of 60 s at 72°C, 60 s at 94°C, and 60 s at 54°C; and 1 cycle of 10 min at 72°C; P2, 1 cycle of 5 min at 94°C and 2 min at 54°C; 30 cycles of 2 min at 72°C, 1 min at 94°C, and 1 min at 54°C; and 1 cycle of 10 min at 72°C); P3, 1 cycle of 5 min at 94°C and 2 min at 50°C; 30 cycles of 40 s at 72°C, 50 s at 94°C, and 50 s at 50°C; and 1 cycle of 10 min at 72°C.

TABLE 2 Relevant characteristics of the 24 LS_A strains

Strain (hospital[a])	Species[b]	Antibiotype[c]	MLS genotype[d]	CLI		PT	
				MIC (μg/ml)	Susceptibility[e]	MIC (μg/ml)	Susceptibility
S3 (A)	*S. aureus*	Oxa^r Kan^r Tob^r Pef^r Lin^i	*vga*(A) allele 1	0.25	S	0.75	S
S4 (A)	*S. aureus*	Oxa^r Kan^r Tob^r Pef^r Lin^i	*vga*(A)v	0.25	S	0.75	S
S5 (A)	*S. aureus*	Oxa^r Kan^r Tob^r Gen^r Pef^r Sul^r Rif^r Lin^r	*vga*(A)v	1	R	0.5	S
S6 (E)	*S. aureus*	Oxa^r Kan^r Tob^r Pef^r Lin^i	*vga*(A)v	0.25	S	0.5	S
S7 (D)	*S. epidermidis*	Pen^r Lin^r	*vga*(A) allele 2	0.75	R	0.19	S
S8 (B)	*S. aureus*	Oxa^r Pef^r Lin^i	*vga*(A)v	0.25	S	0.38	S
S9 (B)	*S. aureus*	Oxa^r Tet^r Sxt^r Lin^i	*vga*(A)v	0.5	I	0.5	S
S10 (B)	*S. aureus*	Oxa^r Kan^r Tob^r Pef^r Lin^i	*vga*(A)v	0.25	S	0.5	S
S11 (B)	*S. aureus*	Pen^r Lin^r	*lnu*(A) + *vga*(A)	0.19	S	0.38	S
S12 (C)	*S. aureus*	Oxa^r Kan^r Tob^r Pef^r Lin^i	*vga*(A)v	0.25	S	0.5	S
S13 (B)	*S. aureus*	Pen^r Kan^r Tob^r Tet^r Sxt^r Lin^r	*lnu*(A) + *vga*(A)	0.19	S	0.38	S
S14 (A)	*S. sciuri*	Lin^i	Unknown	0.38	S	0.75	S
S15 (E)	*S. epidermidis*	Oxa^r Kan^r Tob^r Gen^r Pef^r Sxt^r Lin^i	*vga*(A)v	0.5	I	0.75	S
S17 (C)	*S. aureus*	Oxa^r Pef^r Sul^r Lin^i	*vga*(A)	0.19	S	0.5	S
S19 (C)	*S. epidermidis*	Oxa^r Kan^r Tob^r Gen^r Pef^r Sxt^r Lin^i	*vga*(A)v	0.5	I	0.5	S
S22 (E)	*S. aureus*	Oxa^r Kan^r Tob^r Gen^r Pef^r Sxt^r Lin^i	*vga*(A)v	0.5	I	0.5	S
S23 (E)	*Staphylococcus hominis*	Oxa^r Kan^r Tob^r Gen^r Pef^r Sxt^r Tei^r Lin^i	*vga*(A)	0.25	S	0.5	S
S25 (D)	*S. aureus*	Oxa^r Kan^r Tob^r Pef^r Lin^i	*vga*(A)v	0.25	S	0.75	S
S26 (D)	*S. epidermidis*	Oxa^r Kan^r Tob^r Gen^r Pef^r Sxt^r Lin^i	*vga*(A)v	0.5	I	1	S
S27 (B)	*S. aureus*	Oxa^r Kan^r Tob^r Pef^r Lin^i	*vga*(A)v + *vat*(A)	0.25	S	12	R
S29 (B)	*S. aureus*	Oxa^r Kan^r Tob^r Pef^r Lin^r	*vga*(A)v	0.5	I	1	S
S31 (C)	*S. haemolyticus*	Fos^r Lin^r	$vga(A)_{LC}$	3	R	0.38	S
S33 (A)	*S. epidermidis*	Oxa^r Kan^r Tob^r Gen^r Pef^r Sxt^r Lin^i	*vga*(A)v	0.5	I	0.75	S
S36 (E)	*S. epidermidis*	Oxa^r Kan^r Tob^r Gen^r Pef^r Sxt^r Fus^i Lin^i	*vga*(A)v	0.5	I	0.5	S

[a] Hospitals at which strains were collected: A, HIA Val-de-Grace, Paris, France; B, HIA Robert Picqué, Bordeaux, France; C, HIA Clermont-Tonerre, Brest, France; D, HIA Percy, Clamart, France; E, HIA Legouest, Metz, France.
[b] Species identification was done by 16S rRNA gene sequencing.
[c] Antibiotype was determined according to EUCAST and CA-SFM table breakpoints. Fos, fosfomycin; Fus, fusidic acid; Gen, gentamicin; Kan, kanamycin; Lin, lincomycin; Oxa, oxacillin; Pef, pefloxacin; Pen, penicillin; Rif, rifampin; Sul, sulfonamide; Sxt, co-trimoxazole; Tei, teicoplanin; Tet, tetracyclin; Tob, tobramycin.
[d] The macrolide-lincosamide-streptogramin (MLS) genotype was obtained by PCR amplification and direct sequencing.
[e] S, susceptible; R, resistant; I, intermediate.

most prevalent gene is disseminated and resistance levels are conferred by a series of Vga(A) isoforms detected in a 3-month sampling procedure.

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Screening for the LS_A phenotype was initially carried out at five French military hospitals among clinical isolates identified to be erythromycin-susceptible (Ery^s) staphylococci. This was done by disk diffusion susceptibility tests which incorporated lincomycin as the best indicator of lincosamide resistance (10). The susceptible/intermediate/resistant categorization for the 14 other tested antibiotics was obtained using a SIRscan automated system and EUCAST interpretive criteria (11). For lincomycin, breakpoint values were provided by the Comité de l'Antibiogramme de la Société Francaise de Microbiologie (CA-SFM). Strains with diameters of less than 17 mm were defined as resistant, while those with diameters equal to or greater than 17 mm but less than 21 mm were considered intermediate. Following these French guidelines, 36 lincomycin-resistant (Lin^r) or lincomycin-intermediate (Lin^i) strains were collected and included in the study for further characterization.

Bacterial DNA of the 36 screened strains was extracted using a MagNA Pure LC DNA isolation kit III on a MagNA Pure LC instrument (Roche Diagnostics) and eluted in 50 μl of elution buffer. Various DNA targets were amplified using different PCR primer sets already described or specifically designed for this study (Table 1). Accurate species identification was provided by rRNA gene sequencing as described previously (12) by running the SeqMatch algorithm (http://rdp.cme.msu.edu) for taxonomic assignment. Detection of genes coding for lincomycin resistance was positive for all but one strain. The only negative sample corresponded to a *Staphylococcus sciuri* strain. A total of 10 putative duplicates, which either were from the same patient or shared the same antibiotype at a given hospital, were excluded.

The 26 remaining strains were tested for growth on brain heart

infusion plates supplemented with 6 μg/ml pristinamycin IIA and replica plated with 2 μg/ml lincomycin for storage. Only two of them did not grow on test plates, demonstrating that a lack of susceptibility to lincomycin in Ery^s staphylococci, at least in France, is often linked to co- or cross-resistance to S_A compounds. These two strains harbored an *lnu*(A) gene and thus exhibited the L phenotype, but not the LS_A phenotype. The MICs of clindamycin (CLI) and pristinamycin (PT) for the 24 strains displaying the LS_A phenotype were determined using Etest strips (AB Biodisk) (Table 2). According to identical EUCAST and CA-SFM breakpoint tables, only 1 strain was found to be resistant to PT (MIC > 2 μg/ml), while 12 others were defined to be resistant to CLI (with 3 having MICs of >0.5 μg/ml) or intermediate (with 9 having MICs of 0.5 μg/ml). Once again and unlike the findings for *Enterococcus faecalis*, it was verified that the LS_A phenotype detected among staphylococci did not impair the efficacy of the streptogramin mixtures typified by PT.

Except for the *S. sciuri* strain, all 23 other strains were PCR positive for *vga*(A) detection using primers V1 and V2. To analyze allele diversity within *vga*(A) in the linker region known to be important for antibiotic substrate specificity (6), 900 bp of each amplicon was sequenced with both or either of the two PCR primers. Comparison with protein sequences in the GenBank database yielded the following results: 4 linkers were exactly the same as Vga(A), 16 linkers were exactly the same as the Vga(A) variant [Vga(A)v], 1 linker was exactly the same as $Vga(A)_{LC}$, 1 linker (allele 1) had two of the four mutations (L212S and A220T) reported for $Vga(A)_{LC}$ plus another newly described one (G215S), and 1 linker (allele 2) was exactly the same as EES57021, for which no functional information is available in the literature.

Using primers X and Y (Table 1), which point away from the EES57021 linker sequence, an inverse PCR cloning procedure was conducted to obtain the whole sequence of this *vga*(A) variant [*vga*(A)v] gene, along with additional information about its flanking regions. After digestion of bacterial DNA with HpaII, the corresponding restricted fragments were ligated and subjected to PCR amplification. The UV agarose-detectable amplicon was cleaned up by use of a Qiagen QIAquick PCR purification kit, cloned into a pCR4-Blunt TOPO vector (Invitrogen), and subsequently transformed into chemically competent *Escherichia coli* TOP10 cells. Completion of the sequence of the DNA insert was obtained using M13 universal and reverse primers, as well as primers X and Y. The 3.05-kbp sequence identified the EES57021 *vga*(A) variant in a gene linkage to be identical to that recently described for mobilizable plasmid pUR3036 (13).

To gain information on the functionality of EES57021 gene, plasmid DNA of strain S7 was isolated according to a modified Qiagen alkaline lysis procedure (14). The 2.7-kbp PvuII-EcoRI restriction fragment encompassing the presumed resistance gene was then cloned into SmaI-EcoRI-digested shuttle vector pRB474 (15). The recombinant plasmid was electroporated into *Staphylococcus aureus* RN4220. In comparison with *S. aureus* RN4220 carrying the empty plasmid, *S. aureus* RN4220 transformants carrying the EES57021 *vga*(A) variant cloned into pRB474 exhibited 4-fold- and 32-fold increased MIC values of clindamycin (1 μg/ml) and pristinamycin IIA (32 μg/ml), respectively. It would be useful to evaluate whether this variant confers resistance to pleuromutilin antibiotics.

Following functional analysis of the EES57021 gene, nine Vga(A) variant proteins were characterized for their resistance properties (6, 13, 16–18). Their phylogenetic relationships distinguished Vga(A)v from all others (Fig. 1). The *vga*(A)v gene was shown to be disseminated by a chromosomal transposon rather than by plasmids (19, 20). In light of data collected in the United States and Europe, the allelic variability of *vga*(A) seems to be greater when a plasmid constitutes its genetic support. Most of the Vga(A) protein polymorphism was located within 20 amino acids of the linker region. This is clearly a hot spot for modulating the substrate specificity of the Vga(A) ABC proteins. There would appear to be an increased level of lincosamide resistance when leucine, glycine, and alanine residues of the Vga(A) allele linker sequence are mutated to serine or threonine residues. This was demonstrated by the study of Novotna and Janata in 2006 with $Vga(A)_{LC}$ (6) and by our present results with EES57021. Of note, the S7 strain of *Staphylococcus epidermidis* that bears an EES57021-carrying plasmid was one of the three clindamycin-resistant strains: the two others were the S5 strain of *S. aureus*, which carries a *vga*(A)v gene, and the S31 strain of *Staphylococcus haemolyticus*, which carries a $vga(A)_{LC}$ gene. The infrequent and confirmed plasmid location of the *vga*(A)v gene within strain S5 might explain why the levels of clindamycin resistance are higher than those usually observed for the other isolates (Table 2).

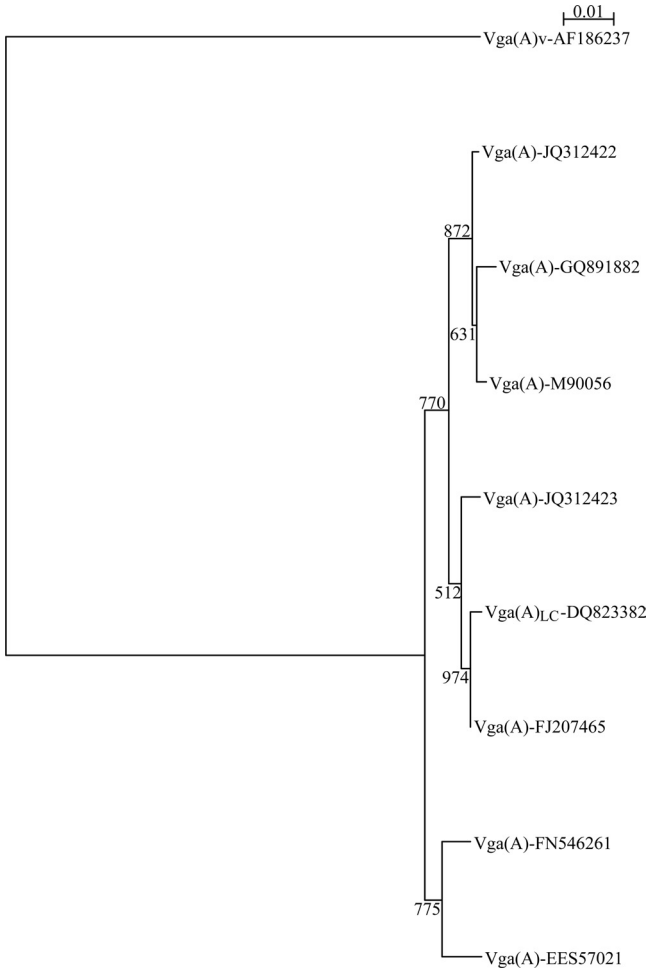

FIG 1 Phylogenetic relatedness among Vga(A) isoforms. Multiple-sequence alignment was carried out with the ClustalX program. The phylogenetic tree was constructed using bootstrap neighbor joining and visualized by NJ plot tree drawing software.

A Tn*5406* transposon structure was detected by PCR using primers T1 and T2 for all *vga*(A)v-carrying strains (Tables 1 and 2). To the best of our knowledge, this is the first report of the presence of Tn*5406* within *S. epidermidis* isolates. Even though several PCR assays using various control strains and validated primers have been performed (21–24), no known resistance gene responsible for the LS_A phenotype was evidenced in the unique *S. sciuri* strain. Since the time of this study, only one other possible resistance gene has been described among staphylococci. It corresponds to *lsa*(E) (25). This gene was found to be in close association with *lnu*(B) on plasmids that disseminate LS_A resistance. If present in *S. sciuri*, the novel gene would be disseminated alone or in a remote gene linkage. Indeed, no trace of DNA homologous to *lnu* sequences (Table 1) was evidenced, apart from *lnu*(A) (Table 2).

In conclusion, this study does not show a wide diversity of resistance genes underlying the LS_A phenotype among French staphylococcal isolates. The *vga*(A)v gene was found to be the most prevalent, and Tn*5406* was first detected in *S. epidermidis*. Due to its chromosomal location, this resistance gene may not evolve as rapidly as the other *vga*(A) variant genes.

Nucleotide sequence accession number. The 3.05-kbp sequence was deposited in GenBank under accession number KC539823.

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