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Differences in Potential for Selection of Clindamycin-Resistant Mutants Between Inducible *erm*(A) and *erm*(C) *Staphylococcus aureus* Genes▽

Claire Daurel,[1,2] Corinne Huet,[2,3] Anne Dhalluin,[2,4] Michèle Bes,[5] Jerome Etienne,[5] and Roland Leclercq[1,2]*

Service de Microbiologie, CHU Côte de Nacre,[1] and EA 2128 Interactions hôte et microorganismes des épithéliums[2] and UFR Sciences Pharmaceutiques,[4] Université de Caen Basse-Normandie, 14033 Caen cedex, CH Louis Pasteur, Cherbourg, 50100,[3] and INSERM U851, Centre National de Référence des Staphylocoques, Faculté Laennec, Université Lyon 1, 69008 Lyon,[5] France

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In staphylococci, inducible macrolide-lincosamide-streptogramin B (MLS_B) resistance is conferred by the *erm*(C) or *erm*(A) gene. This phenotype is characterized by the erythromycin-clindamycin "D-zone" test. Although clindamycin appears active in vitro, exposure of MLS_B-inducible *Staphylococcus aureus* to this antibiotic may result in the selection of clindamycin-resistant mutants, either in vitro or in vivo. We have compared the frequencies of mutation to clindamycin resistance for 28 isolates of *S. aureus* inducibly resistant to erythromycin and bearing the *erm*(C) ($n = 18$) or *erm*(A) ($n = 10$) gene. Seven isolates susceptible to erythromycin or bearing the *msr*(A) gene (efflux) were used as controls. The frequencies of mutation to clindamycin resistance for the *erm*(A) isolates (mean ± standard deviation, $3.4 \times 10^{-8} \pm 2.4 \times 10^{-8}$) were only slightly higher than those for the controls ($1.1 \times 10^{-8} \pm 6.4 \times 10^{-9}$). By contrast, *erm*(C) isolates displayed a mean frequency of mutation to clindamycin resistance ($4.7 \times 10^{-7} \pm 5.5 \times 10^{-7}$) 14-fold higher than that of the *S. aureus* isolates with *erm*(A). The difference was also observed, although to a lower extent, when *erm*(C) and *erm*(A) were cloned into *S. aureus* RN4220. We conclude that *erm*(C) and *erm*(A) have different genetic potentials for selection of clindamycin-resistant mutants. By the disk diffusion method, *erm*(C) and *erm*(A) isolates could be distinguished on the basis of high- and low-level resistance to oleandomycin, respectively.

Clindamycin is an alternative drug for infections due to *Staphylococcus aureus* in case of intolerance to penicillins or resistance to methicillin. Furthermore, clindamycin represents an attractive option for several reasons. First, clindamycin is available in both intravenous and oral formulations. Second, the drug has a remarkable distribution into skin and skin structures. Third, community-acquired methicillin-resistant *S. aureus* (CA-MRSA), which has rapidly emerged in recent years as a cause of skin and soft-tissue infections, is frequently susceptible to several antibiotics, including clindamycin (12, 19). Finally, it has been shown that clindamycin inhibits the production of toxins and virulence factors in gram-positive organisms through inhibition of protein synthesis (7).

However, resistance to clindamycin, which is not rare, limits the use of this antibiotic in therapy. Two primary mechanisms result in resistance to macrolide antibiotics in staphylococci: macrolide efflux, controlled by the *msr*(A) gene, and modification of the drug-binding site on the ribosome, controlled by *erm* (erythromycin ribosome methylation) genes (9). The efflux mechanism yields inducible resistance to 14-membered (erythromycin, clarithromycin, roxithromycin) and 15-membered (azithromycin) macrolides and type B streptogramins but not to lincosamides (clindamycin and lincomycin). Ribosomal methylation confers cross-resistance to macrolides, lincosamides, and type B streptogramins, the so-called MLS_B phenotype. In staphylococci, *erm*(A) or *erm*(C) is responsible for this cross-resistance phenotype by controlling the methylation of the 23S rRNA binding site of adenosine 2058 (A2058) (*Escherichia coli* numbering). Methylation results in impaired binding of the three drug classes that share this residue as a common binding site.

MLS_B resistance can be expressed either constitutively or inducibly. Strains with constitutive resistance express cross-resistance to MLS_B antibiotics. Strains with inducible MLS_B resistance (MLS_Bi) display in vitro resistance to 14- and 15-membered macrolides, which are inducer antibiotics, while appearing susceptible to lincosamides and type B streptogramins, which are not inducers. In the absence of an inducer, the inactivity of the mRNA is due to the structure of its 5′ end, which comprises a leader peptide and a set of inverted repeats that form a hairpin structure which sequesters the initiation sequences (ribosome binding site and initiation codon) for the methylase by base pairing. According to the model of posttranscriptional regulation, induction arises through binding of an inducer macrolide to a ribosome during translation of the leader peptide, leading to destabilization of the hairpin structure, exposure of the initiation sequences to the ribosome, and translation of the Erm methylase (2).

Although clindamycin is not an inducer, exposure of MLS_B-inducible *S. aureus* to this antibiotic may result in cross-MLS_B resistance, either in vitro or in vivo. This is due to the selection of preexisting constitutive *erm* mutants (4, 6, 17). Whether in laboratory strains or in clinical isolates, deletion of the entire attenuator, point mutations, or tandem duplications in the attenuator yield constitutive expression of resistance by de-

* Corresponding author. Mailing address: CHU de Caen, Service de Microbiologie, Avenue Côte de Nacre, 14033 Caen Cedex, France. Phone: (33) 02 31 06 45 72. Fax: (33) 02 31 06 45 73. E-mail: leclercq-r@chu-caen.fr.

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TABLE 1. Primers used in this study

Gene	Primer designation	Primer sequence (5′ to 3′)[a]	Product size (bp)
For cloning into *S. aureus* RN4220			
erm(A)	*erm*(A)BH	+ CAGTGGATCCTCTTATCAAG	1,107
	erm(A)XB	− CTTGTGATCTAGAGAACGC	
erm(C)	*erm*(C)BH	+ TTAGATGGATCCCTCATATC	999
	erm(C) XB	− TGCATCTCTAGACTTACTTATT	
For sequencing of the translational attenuator			
erm(A)	*erm*(A)1LP	+ TTTTTAGTAAAGACAGTGGC	481
	erm(A)2LP	− GTCCTTTTCCTGATCCGA	
erm(C)	*erm*(C)1LP	+ TACAAGAAAAAAGAAATTAG	327
	erm(C)2LP	− ATCTATATTATGTTTTGAAG	

[a] +, sense primer; −, antisense primer.

creasing the stability of the hairpin structure sequestering the initiation sequences for the methylase.

Clinical failures related to the selection of clindamycin-resistant mutants have been reported in a few cases, and there is concern over the emergence of resistance during clindamycin therapy (4, 6, 10, 16, 17). The risk for selection of resistance may depend on various factors, including the frequency of mutation to resistance, the bacterial inoculum size, and the type of infection. Since *erm*(A) and *erm*(C) attenuators display marked differences in terms of length and of leader peptides and inverted-repeat sequences, we hypothesized that the clindamycin mutation frequencies and therefore the risk of mutant selection might differ according to the gene.

In this study, we compare the clindamycin mutation frequencies of clinical isolates of *S. aureus* containing an *erm*(A) or an *erm*(C) gene and propose the oleandomycin test as a marker to identify the genotype of the isolate.

MATERIALS AND METHODS

Bacterial strains and antibiotic susceptibility testing. Twenty-eight *S. aureus* isolates inducibly resistant to erythromycin and bearing the *erm*(C) ($n = 18$) or *erm*(A) ($n = 10$) gene were included in the study. Twelve of these isolates [five *erm*(A) isolates, kindly provided by Peter Appelbaum, and seven *erm*(C) isolates] were CA-MRSA isolates representative of European and North-American clones. Four *erm*(A) and seven *erm*(C) isolates were hospital-acquired MRSA isolates. The other isolates were susceptible to methicillin.

Three erythromycin- and clindamycin-susceptible *S. aureus* isolates (one clinical isolate, *S. aureus* RN4220, and *S. aureus* ATCC 29213), and four *S. aureus* isolates with the efflux gene *msr*(A) were included as controls. *Escherichia coli* DH10B and *S. aureus* RN4220 were used as recipients for cloning experiments.

Susceptibility to antibiotics was determined by the agar diffusion technique with disks (Bio-Rad, Marnes-la-Coquette, France) of erythromycin (15 μg), oleandomycin (15 μg), and clindamycin (2 μg) as recommended by the Comité de l'Antibiogramme de la Société Française de Microbiologie (CA-SFM) (1). Briefly, a suspension in 0.9% saline equivalent to a 0.5 McFarland standard was prepared from an overnight agar medium culture plate, diluted to 1/100 (~10^6 CFU/ml), and spread by swabbing onto Mueller-Hinton agar. We confirmed the inducible phenotype by the D-zone test, which consists of placing standard erythromycin and clindamycin disks in adjacent positions 15 to 26 mm apart on a Mueller-Hinton agar plate as recommended by the Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) (14). Inducible resistance to clindamycin is expressed as a flattening of the clindamycin zone of inhibition adjacent to the erythromycin disk, giving a D shape to the zone of inhibited growth following 16 to 18 h of incubation at 35°C in ambient air.

Mutation frequencies. For determination of mutation frequencies, cells from an overnight agar culture were suspended in saline to a turbidity of a 6 MacFarland standard, and ca. 10^8 to 5×10^9 CFU was spread onto Trypticase-soy agar plates (Bio-Rad) supplemented with 20 μg/ml of clindamycin. The bacterial inoculum was measured by using a spiral system (Interscience, Saint-Nom-la-Bretèche, France). After 48 h of incubation at 35°C, colonies were counted and the mutation frequencies were determined relative to the total count of viable organisms plated. Each experiment was repeated three or four times, and results were expressed as means and standard deviations.

The presence of hypermutable strains among the *S. aureus* strains studied was searched for by spreading ca. 10^7 to 10^8 CFU on Trypticase soy agar plates supplemented with rifampin (100 μg/ml) (Sigma-Aldrich, Saint Louis, MO). After 48 h of incubation at 35°C, every isolate that yielded >10 colonies on antibiotic-containing medium was considered a potential hypermutable bacterium (15). The experiment was repeated in triplicate.

The means were compared by the parametric *t* test and the nonparametric Mann-Whitney test. All *P* values were based on two-sided comparisons and were taken to be significant at ≤0.05 (5).

Molecular techniques. DNA was extracted by the Instagen Matrix kit (Bio-Rad) as recommended by the manufacturer. The presence of the methylase genes *erm*(A) and *erm*(C) and of the efflux gene *mrs*(A) in the isolates studied was confirmed by PCR as described previously (11).

Sequences upstream from the methylase genes of 18 clindamycin-resistant mutants, 9 derived from *S. aureus* strain 23 [a clinical isolate containing the *erm*(A) gene] and 9 derived from *S. aureus* strain 9 [a clinical isolate containing the *erm*(C) gene] were amplified by PCR with the oligonucleotides shown in Table 1. The PCR consisted of 30 cycles of denaturation (95°C, 30 s), annealing [50°C, 30 s, for *erm*(A) and 48°C, 30 s, for *erm*(C)], and extension (72°C, 1 min). Amplified DNA strands were sequenced in both directions. DNA analyses and nucleotide comparisons were carried out using the National Center for Biotechnology Information server at http://www.ncbi.nlm.nih.gov or the MultAlin server at http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html.

Cloning experiments. The entire *erm*(A) and *erm*(C) genes were amplified from two clinical isolates with the specific primers shown in Table 1 and were cloned into the multicopy shuttle vector pAT28 (spectinomycin resistance) using the BamHI and XbaI restriction sites (18). The plasmid constructs were introduced into *E. coli* DH10B by electroporation and were subsequently extracted and electroporated into *S. aureus* RN4220. Staphylococcal transformants were selected on Trypticase soy agar plates containing 60 μg/ml of spectinomycin and 50 μg/ml of erythromycin. The MLS_B resistance phenotype of two transformants, called *S. aureus* RN4220erm(A) and RN4220erm(C), was verified by the D-zone test, and mutation frequencies on clindamycin were determined as mentioned above.

RESULTS

Phenotypes and mutation frequencies. The inducible MLS_B phenotype was confirmed for the *erm*(A) and *erm*(C) isolates by the D-zone test. Notably, no inhibition zone was observed with the oleanomycin disk for *erm*(C) isolates, whereas *erm*(A) isolates displayed zone diameters greater than 15 mm (range, 16 mm to 21 mm).

No clindamycin-resistant mutant could be selected for *S. aureus* ATCC 29213, *S. aureus* RN4220, or one *erm*(A) and three *msr*(A) isolates by using inocula between 1.2×10^8 and 2.5×10^9 CFU. For those strains that did not yield mutants,

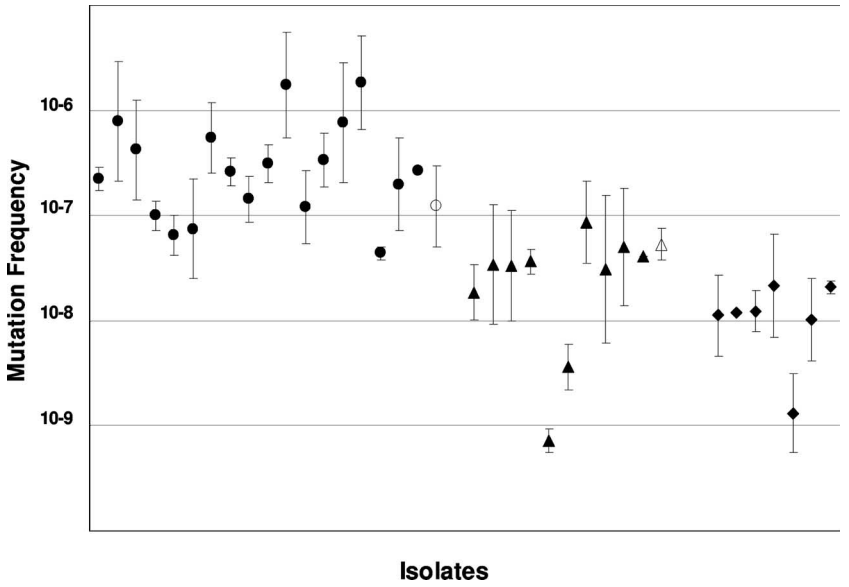

FIG. 1. Frequencies of mutation to clindamycin resistance of *S. aureus* strains, including 18 with *erm*(C) (solid circles), 10 with *erm*(A) (solid triangles), and 7 controls (solid diamonds). Open circle and triangle represent RN4220erm(C) and RN4220erm(A), respectively. Approximately 10^8 to 5×10^9 CFU of cells was spread onto agar plates containing 20 μg/ml of clindamycin. After 48 h of incubation at 35°C, colonies were counted, and the mutation frequencies were determined relative to the total count of viable organisms plated. Each data point represents the mean mutation frequency calculated from three experiments for one strain.

the inverse of the inoculum value was taken as the frequency of mutation for the purpose of calculations.

The frequencies of mutation to clindamycin resistance for the *erm*(A) isolates (range, 7.3×10^{-10} to 1.8×10^{-8}; mean ± standard deviation, $3.4 \times 10^{-8} \pm 2.4 \times 10^{-8}$) were slightly higher than those for the controls (range, 1.3×10^{-9} to 1×10^{-8}; mean ± standard deviation, $1.1 \times 10^{-8} \pm 6.4 \times 10^{-9}$) (Fig. 1). However, the difference was significant by the *t* test ($P = 0.046$) but not by the Mann-Whitney test ($P = 0.055$).

By contrast, mutants were readily obtained for all the *S. aureus* isolates with *erm*(C). On average, *erm*(C) isolates displayed a 14-fold higher frequency of mutation to clindamycin resistance (range, 1.7×10^{-6} to 4.4×10^{-8}; mean ± standard deviation, $4.7 \times 10^{-7} \pm 5.5 \times 10^{-7}$) than the *S. aureus* isolates with *erm*(A) (Fig. 1). These differences were observed whether the isolates were susceptible or resistant to methicillin. The means of mutation frequencies in the *erm*(C) group differed significantly from those in the *erm*(A) and control groups by the Mann-Whitney test ($P < 0.001$ and $P = 0.002$, respectively) and by the *t* test ($P = 0.019$ and $P = 0.040$, respectively). Therefore, clinical strains of *S. aureus* with the *erm*(C) gene are the most likely to readily develop constitutive resistance to clindamycin.

We could not exclude the possibility that the higher mutation frequencies observed for the *erm*(C) isolates might be related to a hypermutable background of the isolates. Therefore, we screened for a putative hypermutable status of those isolates. No potential hypermutable bacteria were detected, and no difference could be observed between *erm*(A) and *erm*(C) isolates. In addition, no clear difference between mutation rates for hospital-acquired MRSA versus CA-MRSA *erm*(C) and *erm*(A) isolates was observed.

The introduction of *erm*(C) and *erm*(A) genes into *S. aureus* RN4220 resulted in differences in frequencies of mutation to clindamycin resistance with, again, a higher frequency for *erm*(C) [$1.2 \times 10^{-7} \pm 2.1 \times 10^{-7}$ for *erm*(C) versus $5.3 \times 10^{-8} \pm 3.6 \times 10^{-8}$ for *erm*(A)]. This observation confirmed the data for clinical isolates, although the difference was less striking.

Methylase attenuator mutations. The sequence of the attenuator was compared between MLS_{Bi} parents and derivatives. Structural alterations in the attenuators of four of nine clindamycin-resistant derivatives of *S. aureus* strain 9 [*erm*(C)] were observed. Two deletions of 15 and 16 bp, one deletion of 50 bp, and one tandem duplication of a 16-bp sequence resulted in marked alterations in a series of inverted repeats involved in the control of methylase expression (Fig. 2). In all cases, alterations of the attenuator would potentially lead to constitutive expression of the methylase, whether under the control of the ribosome binding site of the leader peptide or of its own ribosome binding site. In particular, the 50-bp deletion would delete the stop codon of the leader peptide, creating a translational fusion of the peptide with the methylase.

By contrast, none of the nine *erm*(A) mutants analyzed contained any alteration of the regulatory region. This unexpected result might be related to the use of a relatively low inoculum (approximately 10^9 CFU), which might be insufficient to select mutants with alterations of the *erm*(A) attenuator in *S. aureus* 23 but enough to select ribosomal mutants.

DISCUSSION

Clindamycin is widely used to treat serious staphylococcal infections, in particular for children, due to limited alternative therapy. However, recent reports indicate that failure may occur in the case of inducible MLS_B resistance in spite of in

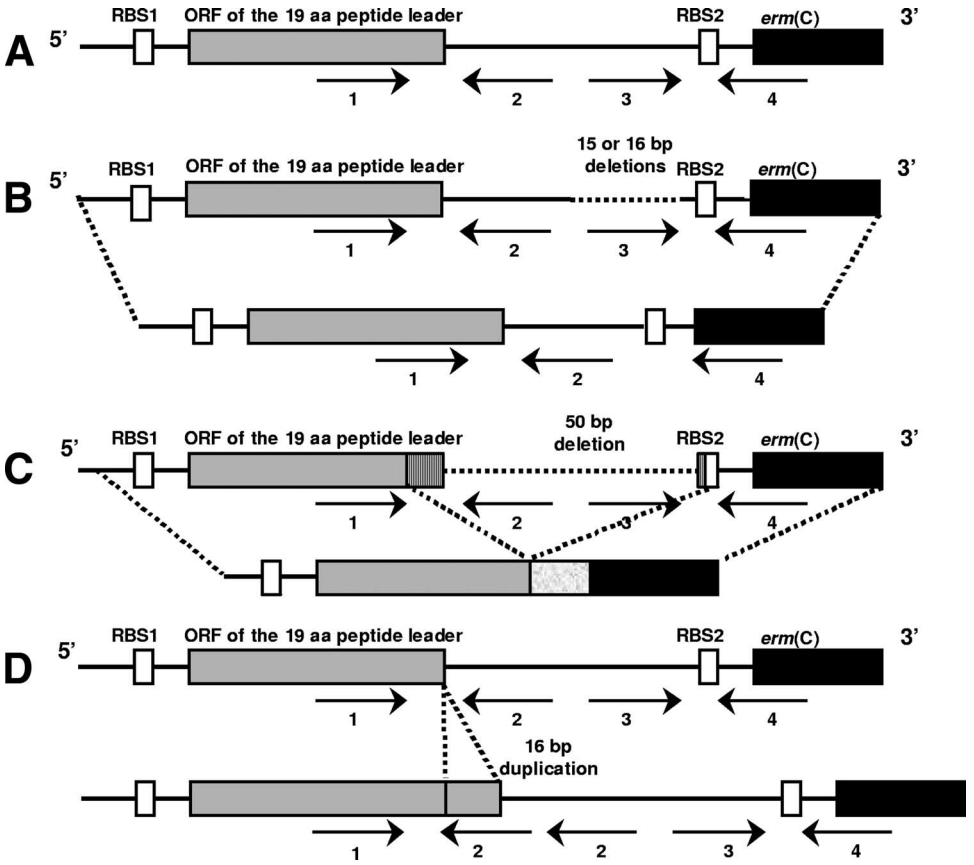

FIG. 2. Schematic presentation of the regulatory regions of the inducibly expressed *erm*(C) gene from *S. aureus* 9 (A) and the constitutively expressed *erm*(C) genes selected in this study (B, C, and D). RBS1 and RBS2, ribosome binding sites of the leader peptide and the *erm*(C) gene, respectively. Arrows indicate the inverted repeated sequences IR1 to IR4. (A) Wild-type *erm*(C) gene; (B) derivatives with 15- and 16-bp deletions in IR3; (C) derivative with a 50-bp deletion (deletion of the stop codon of the leader peptide generates a translational fusion with the methylase); (D) derivative with tandem duplication of a 16-bp sequence (insertion of a new IR2).

vitro susceptibility to clindamycin (4, 6, 10, 16, 17). In most cases, failure was related to the selection of MLS_B constitutive mutants resistant to clindamycin. Although clinical data are limited and sometimes conflicting, concern over the emergence of resistance during clindamycin therapy has led the CLSI to recommend the use of a simple laboratory test, the erythromycin-clindamycin D-zone test, to differentiate isolates that have a high genetic potential to become resistant during clindamycin therapy (those with *erm* genes) from isolates that have a low genetic potential to become resistant [those with the *msr*(A) gene]. In the study by Lina et al., 70% of MLS_Bi *S. aureus* isolates contained *erm*(C) and 30% contained *erm*(A) (11). In a recent study, among 402 *S. aureus* isolates, the overall prevalence of MLS_Bi was 52%, with 50% of MRSA and 60% of methicillin-susceptible *S. aureus* isolates exhibiting MLS_Bi (12). CA-MRSA strains had a lower prevalence of MLS_Bi than hospital-associated MRSA strain (33% versus 55%). Therefore, the MLS_Bi phenotype is common in staphylococci, although less prevalent in CA-MRSA strains.

In this study, we were able to select mutants resistant to clindamycin from most isolates, including those that were susceptible to erythromycin. Generally, frequencies of mutation to clindamycin were around 10^{-8} or below for susceptible isolates. Although we did not study the mechanisms of clindamycin resistance, mutations in ribosomal structures such as the *rrl* gene (23S rRNA) or ribosomal proteins were probably involved. In agreement with this idea, a previous study has shown that selection of ribosomal mutants by erythromycin occurred in erythromycin-susceptible and nonhypermutable *S. aureus* strains at frequencies similar to those determined in our study for clindamycin resistance (15). Mutations to clindamycin resistance were within the same range for isolates with the *msr*(A) gene, confirming that this type of isolate was not particularly at risk for selection of resistant mutants, similarly to wild-type isolates.

The *erm*(A) isolates had mutation frequencies between 10^{-7} and 10^{-8}, slightly higher than those for the *msr*(A) or susceptible isolates. By contrast, mutation frequencies were generally between 10^{-6} and 10^{-7} for *erm*(C) isolates; thus, the mean risk of mutation was 14 times greater than that for the *erm*(A) isolates. The behavior of two strains of *S. aureus* RN4220 that differed only in carrying the *erm*(A) or the *erm*(C) gene confirmed this observation. However, the mutation frequencies for the constructs *S. aureus* RN4220erm(A) and RN4220erm(C) were only slightly different. This could be due to the cloning of *erm*(A) on a multicopy plasmid, whereas under natural conditions the gene is usually borne by transposon Tn*554* and is present in a single copy or a few copies in the chromosome of

S. aureus (13). By contrast, *erm*(C) is often borne by small multicopy plasmids in clinical isolates (8).

The reason for the differences in mutation frequencies between *erm*(A) and *erm*(C) might be explained by differences in the respective structures of attenuators. The regulatory region of *erm*(C) comprises one leader peptide and four inverted repeats. *erm*(A) has a longer regulatory region, with a much more complex structure that includes two leader peptides and six inverted repeats (13). Possibly, due to the complexity of the *erm*(A) attenuator, there are fewer opportunities for mutations spontaneously occurring in the regulatory region to generate constitutive expression of MLS_B resistance than there are for the *erm*(C) attenuator. Alternatively, the particular hairpin structure of the *erm*(C) attenuator might favor errors of the DNA polymerase, particularly deletions, more than the *erm*(A) attenuator would. This would explain why we did not observe attenuator alterations in the nine mutants selected from *S. aureus* 23 [*erm*(A)] at the inoculum that we used. By contrast, in four of nine mutants from *S. aureus* 9, deletions or duplications affected the regulatory region of the *erm*(C) gene.

More clinical isolates should be studied to reinforce the validity of our results. In addition, the clinical relevance of the difference observed in vitro between *erm*(C) and *erm*(A) in the selection of clindamycin resistance remains to be fully established. Since the type of *erm* gene was not reported for the published cases of clindamycin failure, we do not know if *erm*(C) was mostly involved, as expected from our results. However, the frequencies of mutation to clindamycin resistance, which are between 10^{-6} and 10^{-7} for the inducible *erm*(C) staphylococci, can be considered relevant for clindamycin therapy of infections with bacterial inocula usually exceeding 10^7 CFU, such as abscesses requiring drainage, mediastinitis, and some lower respiratory tract infections. In these cases, clindamycin therapy has a high probability of failure and should be avoided. The lower frequencies of mutation observed for inducible *erm*(A) staphylococci do not mean that the use of clindamycin is safe in those cases, and if clindamycin is used for treatment of infections due to such isolates, close follow-up and monitoring for failure or relapse are needed.

Further clinical studies are required to establish whether, for a given MLS_Bi isolate, the identification of the type of the *erm* gene has any importance for decision making if clindamycin therapy is considered. Research protocols should include distinction between *erm*(A) and *erm*(C) by genotypic techniques, such as PCR, and the oleandomycin test as a phenotypic marker. In a previous publication, Di Modugno et al. showed that oleandomycin MICs for staphylococci with inducible *erm*(C) genes were greater than 32 μg/ml, whereas those for staphylococci with inducible *erm*(A) genes were 2 to 8 μg/ml, and they proposed to use high-level resistance to oleandomycin as a marker of inducible *erm*(C) genes (3). In the same study, similar differences in clarithromycin MICs were also observed, and these could also be considered as a suitable marker. We confirmed the conclusions of this study, and we propose the use of a disk of oleandomycin (15 μg) to distinguish staphylococci with inducible *erm*(C) genes (no inhibition zone) from those with inducible *erm*(A) genes (zone diameters greater than 15 mm by the CA-SFM technique).

In conclusion, the D-zone test is useful for identifying the non-MLS_Bi *S. aureus* isolates against which clindamycin can be used safely in therapy. When isolates display the MLS_Bi phenotype, the oleandomycin test may provide additional information for evaluation of the potential therapeutic use of clindamycin. In the future, the development of genetic techniques for routine identification of resistance genes will be helpful in guiding therapy more accurately.

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