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Novel 10-bp deletion in the translational attenuator of a constitutively expressed *erm*(A) gene from *Staphylococcus epidermidis*

Leticia Millán,[1] Pilar Goñi,[1] Paula Cerdá,[1] M. Carmen Rubio,[1,2] Rafael Gómez-Lus[1]*

[1]Department of Microbiology, Zaragoza University School of Medicine, Spain. [2]Microbiology Service, Lozano Blesa University Clinic Hospital, Zaragoza, Spain

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Summary. Alterations in the *erm*(A) regulatory region of six clinical isolates of *Staphylococcus epidermidis* and one of *Staphylococcus haemolyticus* displaying a constitutive resistance phenotype were investigated. A novel deletion of 10 bp with respect to the corresponding sequence of Tn*554* was identified in the attenuator of a constitutively expressed *erm*(A) gene of one of the *S. epidermidis* isolates. Thus far, this is the smallest deletion conferring constitutive resistance in the translational attenuator of *erm*(A) in a naturally occurring *S. epidermidis* strain of human origin. [**Int Microbiol** 2007; 10(2):147-150]

Key words: S*taphylococcus epidermidis* · *erm*(A) regulatory region · deletions · macrolide, lincosamide, streptogramin B (MLS_B) resistance

Introduction

Resistance to macrolide, lincosamide, and streptogramin B antibiotics (MLS_B resistance) in staphylococci is mainly due to the dimethylation of an adenine residue (A2058) located in a highly conserved region of the 23S rRNA [6,24]. In staphylococci, four major methylase gene classes, *erm*(A), *erm*(B), *erm*(C), and *erm*(F), all of which play a role in MLS_B resistance [6,12,23], have been identified. Expression of the predominant *erm* genes in staphylococci, *erm*(A) and *erm*(C), is either constitutive or inducible by 14- and 15-membered macrolides. Resistance is not related to the class of the *erm* determinant, but depends on the sequence of the regulatory region upstream of the methylase gene and is explained by a translation attenuation mechanism [6,13,24]. Constitutively expressed *erm*(A) and *erm*(C) genes are of particular clinical relevance since they also confer resistance to 16-membered macrolides, lincosamides, streptogramin B antibiotics, and the new ketolide drugs. However, in inducible MLS_B resistance, the strains are resistant to 14- and 15-membered macrolides only, whereas 16-membered macrolides, lincosamides, streptogramin B antibiotics, and ketolides remain active [6,19]. The *erm*(A) gene, which is part of transposon Tn*554*, is usually integrated at a specific site (*att554*) in the *Staphylococcus aureus* chromosome. However, second insertions have also been described in chromosomal and plasmid sites, both in *S. aureus* and coagulase-negative staphylococci (CoNS) [6,9,10,21,22,25]. Structural alterations in the *erm*(A) regulatory region have been reported for constitutive mutants derived from in-vitro selection experiments and for constitutively expressed naturally occurring staphylococcal plasmids [9,14,25].

In this work, we studied alterations in the regulatory region of the constitutively expressed naturally occurring *erm*(A) gene from six clinical isolates of *Staphylococcus epidermidis* and one clinical isolate of *Staphylococcus haemolyticus*.

***Corresponding author:** R. Gómez-Lus
Department of Microbiology
Zaragoza University School of Medicine
Domingo Miral, s/n
50009 Zaragoza, Spain
Tel. +34-976761692. Fax +34-976761693
E-mail: gomezlus@unizar.es

Materials and methods

Bacterial strains and culture conditions. Six clinical strains of *S. epidermidis* (0467, 4747, 6906, 6926, 7847 and 8377) and one of *S. haemolyticus* (2505) were collected. All strains exhibited a constitutive resistance phenotype and carried the *erm*(A) gene. The strains were isolated from the respiratory tracts of patients in the Microbiology Service of the University Clinical Hospital Lozano Blesa (Zaragoza, Spain) between October 2000 and November 2002. Initial identification was based on colony morphology, microscopy appearance, and agglutination tests with the Pastorex Staph Plus kit (Bio-Rad, La Coquette, France). Species were identified using the WIDER Computerized System (Dade Behring, West Sacramento, CA, USA). The isolates were cultured on Mueller-Hinton agar (MHA) (Bio-Rad, La Coquette, France) at 37ºC and stored frozen (–80ºC) in sterile skimmed milk.

Macrolide-lincosamide resistance testing. All strains had the constitutive resistance phenotype, as confirmed by a triple-disk diffusion technique carried out using a modification of the assay described by Seppälä et al. [18]. The disks contained erythromycin (15 μg), azithromycin (15 μg), clindamycin (2 μg) (Bio-Rad, La Coquette, France), and miocamycin (50 μg, Neo-Sensitabs, Taastrup, Denmark).

PCR amplification. The presence of the *erm*(A), *erm*(B), and *erm*(C) genes was tested by PCR, as reported by Sutcliffe et al. [20]. To detect structural alterations in the *erm*(A) and/or *erm*(C) regulatory regions, the PCR assays described by Werckenthin and Schwarz [25] and by Lodder et al. [8] were used. The expected PCR products were 592 bp for the *erm*(A) regulatory region and 295 bp for that of *erm*(C). All PCR amplifications were carried out in a Perkin-Elmer Cetus DNA Thermal Cycler (Perkin-Elmer, Norwalk, CT, USA), and the products were analyzed by electrophoresis through 1.5% agarose gels. PCR reagents were purchased from Promega (Madison, WI, USA). Three of the PCR amplicons of the *erm*(A) regulatory region were purified (GFX PCR DNA and Gel Band Purification Kit, Amersham Biosciences, Freiburg, Germany) and then sequenced (DYEnamic ET Dye Terminator Cycle Sequencing Kit for MegaBACE DNA Analysis Systems, Amersham). The software BLAST was used to compare the sequences of the PCR products to that of the inducible *erm*(A) gene of Tn*554*, available at the website of the National Center for Biotechnology Information [http://www.ncbi.nlm.nih.gov]. A clinical control strain with an inducible phenotype, *S. aureus* 4850, which harbored *erm*(A) and *erm*(C) with a complete attenuator, was used. The *erm*(A) regulatory region of the control strain was identical to that of Tn*554* (database accession no. X03216), whereas its *erm*(C) regulatory region was identical to that of plasmid pE5 carrying *erm*(C) (database accession no. M17990).

Nucleotide sequence accession number. The nucleotide sequence of the *erm*(A) regulatory region and partial *erm*(A) gene of strain *S. epidermidis* 6926 was submitted to the GenBank database and assigned accession no. DQ112022.

Results and Discussion

Analysis of the presence of the macrolide resistance genes *erm*(A), *erm*(B), and *erm*(C) showed that four *S. epidermidis* (strains 0467, 4747, 6926, and 7847) and one *S. haemolyticus* (strain 2505) harbored both *erm*(A) and *erm*(C), whereas the two remaining *S. epidermidis* isolates (6906 and 8377) carried *erm*(A), *erm*(B), and *erm*(C). Sequence analysis of the PCR fragment obtained from *S. epidermidis* 6926 identified a novel 10-bp deletion in the *erm*(A) regulatory region. In *S. epidermidis* 8377, a 121-bp deletion was observed in that region. The remaining strains (*S. epidermidis* 0467, 4747, 6906, 7847, and *S. haemolyticus* 2505) contained neither deletions nor tandem duplications [14] in the regulatory region. *erm*(A) PCR amplicons of strains *S. epidermidis* 6926, *S. epidermidis* 8377, and *S. epidermidis* 6906 were sequenced.

The 10-bp deletion of *S. epidermidis* 6926 comprised nucleotides 5358–5367 (AGTTCGTTAT), which corresponded to part of the IR3 sequence located in the open reading frame (ORF) of the 19-amino acid (aa) peptide (Fig. 1, database accession no. DQ112022). Since truncated IR3 is not able to form a stable mRNA secondary structure with IR4, the latter will pair with IR5 and thereby render IR6 permanently accessible to ribosomes, allowing constitutive *erm*(A) gene expression to occur. In addition, this strain also harbored an *erm*(C) gene, but sequence analysis of the PCR amplicon corresponding to the gene's regulatory region showed that it shared 100% identity with the regulatory region of the *S. aureus* plasmid pE5, which carries *erm*(C) and confers inducible MLS_B resistance [11] (data not shown). To our knowledge, this is the first report of a 10-bp deletion in the regulatory region of an *erm*(A) gene in a *S. epidermidis* clinical strain with a constitutive resistance phenotype. Schmitz et al. [17] detected a 14-bp deletion that comprised part of the ORF of the 19-aa peptide and part of IR3 (nucleotides 5344–5357). The deletion confers constitutive resistance in *S. aureus* mutants—previously carrying an inducible *erm*(A) gene—selected in vitro in the presence of non-inducer antibiotics. Our work continues and extends this study as it describes a smaller deletion in this region from a naturally occurring *S. epidermidis* strain of human origin, which suggests selective pressure in nature due to the widespread use of MLS_B antibiotics.

The 121-bp deletion of strain *S. epidermidis* 8377 comprised the ORF of the 19-aa peptide as well as the region immediately downstream, which included the IR4 and IR5 sequences. This deletion, which has also been reported by others [14], renders IR6 always accessible to ribosomes, independently of the presence or absence of inducer.

The *erm*(A) regulatory region of *S. epidermidis* 6906 showed complete identity with that of Tn*554*. Contrary to previously described [9,17,25], point mutations that could have accounted for constitutive expression of *erm*(A) were not detected. This strain also harbored *erm*(B) and *erm*(C) determinants. PCR amplification and sequence of the *erm*(C) regulatory region showed complete identity with that of the sequence of plasmid pE5 (data not shown). Thus, constitutive expression of the methylase could be due to *erm*(B), as the latter is located on Tn*551* in staphylococci and expressed constitutively [2]. By contrast, in streptococci and certain

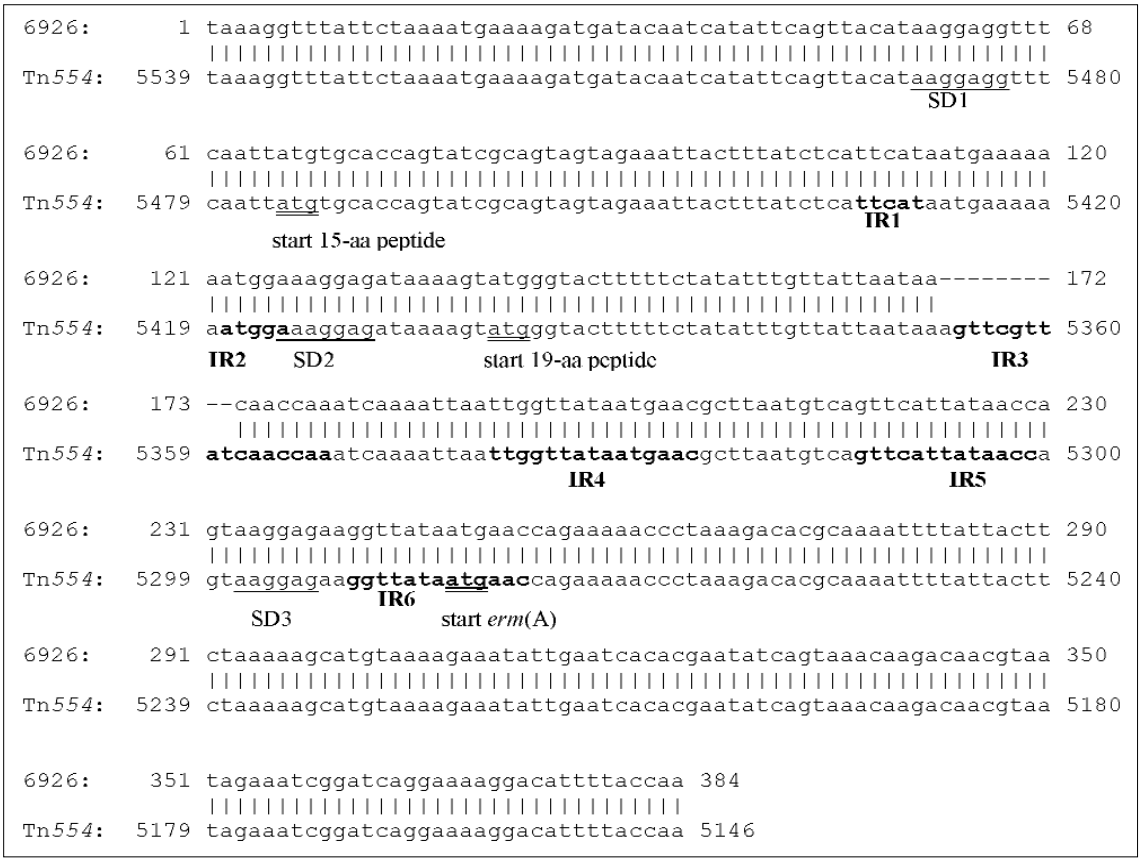

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Fig. 1. Comparison of the regulatory region and the 5′ end of the constitutively expressed *erm*(A) gene of *Staphylococcus epidermidis* 6926 with the corresponding region of Tn554 (database accession No. X03216). Shine-Dalgarno sequences SD1 of the 15-aa peptide, SD2 of the 19-aa peptide, and SD3 of the *erm*(A) gene are underlined. Inverted repeat sequences (IR1 to IR6) are in bold. Start codons of the ORFs of the 15-aa peptide, the 19-aa peptide, and the *erm*(A) gene are double underlined.

other bacteria, *erm*(B) is inducible by the entire range of MLS_B antibiotics, and its regulatory region specifies a 155-bp leader sequence encoding a 36-aa peptide and containing 14 IRs that can potentially form a large number of secondary structures [3,9]. Like the deletions, tandem duplications, and point mutations reported in the regulatory regions of *erm*(A) and *erm*(C) [5,8,9,14,25,26], similar alterations may be present in the *erm*(B) regulatory region of this strain that account for the constitutive phenotype. Furthermore, the *erm*(C) gene of this strain was transferred by filter-mating experiments. The transconjugant obtained presented an inducible phenotype, which supports our hypothesis that constitutive expression is due to *erm*(B) (data not shown). Nevertheless, further sequence analysis of the *erm*(B) regulatory region is currently being done to verify this hypothesis.

The remaining four strains also showed a PCR product whose size was indistinguishable from that of the control strain. In addition, all of them harbored *erm*(C), and analysis of the regulatory region of this gene showed that three strains contained a 107-bp deletion and the other a 58-bp deletion, which could explain the constitutive phenotype (data not shown). These alterations in the attenuator have been described in constitutively expressed *erm*(C) genes of naturally occurring staphylococcal plasmids, such as pSES24 (58-bp deletion), pNE131, and pSES4a (107-bp deletion) [5,8,26], and our strains showed complete identity with them. However, as we did not sequence the translational attenuator of the *erm*(A) gene of these strains, there could be other alterations in this region, such as point mutations, or the 10-bp deletion described in this work (detected only after sequencing), or other deletions of small size [17], which cannot be detected without sequencing. Further studies will seek to determine whether such alterations are present in the *erm*(A) regulatory region of these four strains.

To the best of our knowledge, this is the first description of a 10-bp deletion in the translational attenuator of *erm*(A) from a naturally occurring *S. epidermidis* strain of human origin. It is the smallest of the deletions reported thus far in this region and it confers constitutive resistance. Different mutations in the regulatory regions of *erm*(A) and *erm*(C) occur-

ring in vivo and in vitro after selection in the presence of non-inducer antibiotics have been reported. Non-inducing MLS_B antibiotics are still widely used in human and in veterinary medicine. The development of constitutively resistant strains will strongly reduce the efficacy of these drugs in the control of pathogenic staphylococci. Thus, the spectrum of resistance changes from limited resistance to 14- and 15-membered macrolides to extended resistance to 14-, 15-, and 16-membered macrolides as well as lincosamides and type B streptogramins. Consequently, the use of MLS_B antibiotics is not recommended for the control of staphylococci with an inducible phenotype [4,7–9,14–17,25,26]. Note also that alterations in the regulatory region of *erm*(A) are not only confined to staphylococci; rather, a 44-bp duplication, a 68-bp deletion, point mutations and a single base insertion have also been described in this region in several *Streptococcus pyogenes* clinical isolates displaying constitutive MLS_B resistance [1]. Thus, our results confirm that deletions in the *erm*(A) regulatory region not only are obtained in the laboratory but they also occur in nature, which suggests selective pressure resulting from the use of MLS_B antibiotics.

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