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Naturally Occurring *Staphylococcus epidermidis* Plasmid Expressing Constitutive Macrolide-Lincosamide-Streptogramin B Resistance Contains a Deleted Attenuator

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A naturally occurring constitutive macrolide-lincosamide-streptogramin B (MLS) resistance plasmid, pNE131, from *Staphylococcus epidermidis* was chosen to study the molecular basis of constitutive expression. Restriction and functional maps of pNE131 are presented along with the nucleotide sequence of *ermM*, the gene which mediates constitutive MLS resistance. Sharing 98% sequence homology within the 870-base-pair *Sau*3A-*Taq*I fragment, *ermM* appears to be almost identical to *ermC*, the inducible MLS resistance determinant from *S. aureus* (pE194). The two genes share nearly identical sequences, except in the 5′ promoter region of *ermM*. Constitutive expression of *ermM* is due to the deletion of 107 base pairs relative to *ermC*; the deletion removes critical sequences for attenuation, resulting in constitutive methylase expression.

Staphylococcus epidermidis, normally thought of as a nonpathogenic resident of the human skin and nose, is now recognized as a major cause of infections of prosthetic devices after surgical implantation. Treatment of these infections is often difficult because of the frequent occurrence of multiply antibiotic-resistant strains (1, 16). The presence of resistance plasmids and recent reports of their transfer to the more virulent *S. aureus* have led to speculation that the ubiquitous, more antibiotic-resistant *S. epidermidis* may serve as a reservoir for antibiotic resistance plasmids (2, 25).

We previously described a naturally occurring plasmid, pNE131, in *S. epidermidis* which expresses constitutive resistance to macrolide-lincosamide-streptogramin B (MLS) antibiotics and shares DNA sequence homology with the inducible plasmid pE194 in *S. aureus* (26). Resistance to MLS antibiotics is due to dimethylation of the adenine residue at position 2058 in 23S rRNA (14, 15, 31), resulting in a reduced affinity between ribosomes and antibiotics. Plasmids which mediate MLS resistance encode an *S*-adenosylmethionine-dependent methylase which produces this modification of rRNA (30).

In *Staphylococcus* and *Bacillus* spp., resistance to MLS antibiotics is inducible by a subinhibitory concentration of erythromycin and the closely related macrolide oleandomycin. Several inducible resistance genes have been sequenced and analyzed in molecular detail. The most thoroughly studied is the *ermC* determinant from *S. aureus* (pE194). Inducible expression of this resistance gene has been shown to occur by a posttranscriptional mechanism analogous to transcriptional attenuation in various amino acid biosynthetic operons (5, 10). Similar translational attenuation models have been proposed for other inducible MLS determinants, such as *ermA* from *S. aureus* (23), *ermD* from *Bacillus licheniformis* (6), and *ermAM* from *Streptococcus sanguis* (9).

Although the inducible expression of MLS resistance has been extensively studied, little work has addressed the naturally occurring constitutive MLS resistance plasmids. On the basis of studies presented here and elsewhere (4, 22, 34), the constitutive resistance phenotype appears to be predominant among clinical isolates of *S. epidermidis* and *S. aureus*. In this paper, we present the nucleotide sequence of the MLS resistance gene, *ermM*, from *S. epidermidis* (pNE131). The DNA sequence of this constitutively expressed gene appears to be almost identical to the sequence of the inducible *ermC* gene of plasmid pE194, except for a 107-base-pair (bp) deletion in the 5′ leader region of the mRNA. This deletion removes the various complementary sequences capable of forming the folded mRNA structure characteristic of the attenuator, thus resulting in constitutive expression of MLS resistance.

MATERIALS AND METHODS

Strains and plasmids. Plasmid pNE131 was originally isolated from a clinical culture of *S. epidermidis* obtained at the Health Sciences Center of the University of Missouri—Columbia (26). *S. aureus* RN2442(pE194) was a gift from B. Weisblum. Determination of constitutive or inducible expression of MLS resistance was done by in situ induction on solid medium with Kirby-Bauer antibiotic susceptibility disks as described by Weisblum et al. (35).

Mapping restriction sites. A map of restriction sites for plasmid pNE131 (Fig. 1) was constructed by standard techniques of single and double restriction endonuclease digestions. In addition, *Alu*I and *Sau*3A restriction sites were determined by an end label mapping procedure. Plasmid pNE131 was cleaved at the unique *Hha*I restriction site and labeled at the 5′ terminus with polynucleotide kinase and [γ-^{32}P]ATP as described by Maniatis et al. (17). The plasmid was cleaved again at the *Sst*I site to yield two fragments easily separated by gel electrophoresis and labeled at only one end. Each fragment was then partially digested with *Alu*I or *Sau*3A, electrophoresed in agarose gels, and autoradiographed, and the location of restriction sites was determined as described by Smith and Birnstiel (32).

To map the location of the MLS resistance gene of pNE131 as shown in Fig. 1, we labeled plasmid DNA by nick translation under the conditions described by Maniatis et al. (18) with some modifications, as previously reported (26). Transfer of DNA to nitrocellulose and hybridization of DNA were as previously described (26).

Nucleotide sequencing. Isolation of plasmid DNA for se-

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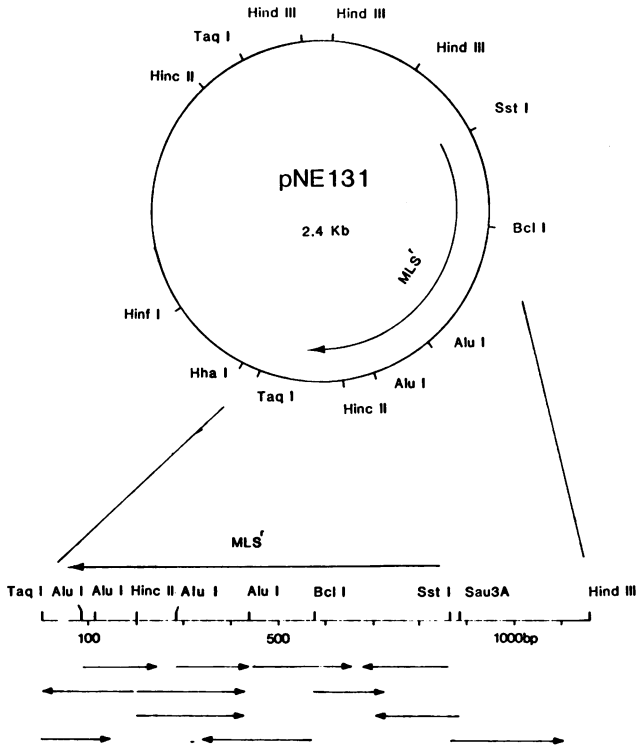

FIG. 1. Restriction and functional maps of pNE131 along with the sequencing strategy used. The large arrow on the circular map indicates the region encoding MLS resistance and the direction of transcription of *ermM*. The bottom of the figure shows the sequencing strategy used. Each arrow under the linear restriction map represents the direction and extent of the nucleotide sequence determined from a given restriction site that was cloned. Kb, Kilobases.

quencing was done by the rapid lysis procedure (13) modified to accommodate large volumes. DNA was purified by cesium chloride-ethidium bromide density gradient centrifugation. Restriction fragments were cloned into the M13 filamentous phage vectors mp18 and mp19 (24) and sequenced by the dideoxy chain termination method of Sanger et al. (28). [α-^{32}P]dATP and 8% acrylamide–8 M urea gels were used. Initially, *Alu*I subfragments of the largest *Taq*I-*Sst*I fragment were "shotgun" cloned into M13 phage vectors and sequenced, followed by a sequence determination from restriction sites of specifically cloned fragments in this region. Except for 96 bp, the entire DNA sequence extending from *Taq*I to *Sst*I was determined from both strands. Overlapping fragments were cloned so that nucleotide sequences would extend across all the cloning sites used.

RESULTS

Restriction and functional maps of pNE131. A partial restriction map of pNE131 is shown in Fig. 1. From an analysis of restriction fragments, the size of pNE131 was estimated to be 2,360 bp. The plasmid contains unique restriction sites for *Hha*I, *Bcl*I, and *Sst*I. A gross comparison of restriction maps between pNE131 and *S. aureus*(pE194) (12) revealed no common pattern of restriction fragments.

To determine the location of the MLS resistance gene on the restriction map of pNE131, we obtained an MLS gene probe from the inducible plasmid pE194. The *Hin*fI A fragment of pE194 (encompassing *ermC*) was radioactively labeled by nick translation and used to probe various restriction fragments of pNE131 in a Southern hybridization experiment (33). Since the largest *Taq*I-*Sst*I fragment of pNE131 hybridized with the probe, the MLS gene was placed on the map of pNE131 as indicated in Fig. 1.

Nucleotide sequence of *ermM*. A linear restriction map of the region of pNE131 which was sequenced along with the sequencing strategy used are shown at the bottom of Fig. 1. The complete sequence of *ermM* is shown in Fig. 2 along with the sequence of the homologous region of the inducible *ermC* gene to illustrate comparisons.

Deletion of the attenuator. A comparison of the DNA sequences of the MLS resistance determinants from *S. epidermidis*(pNE131) and *S. aureus*(pE194) showed that these sequences are nearly identical, except for a 107-bp deletion in the 5′ regulatory region of *ermM*. Among the 870 bp which extend from the *Sau*3A site to the *Taq*I site at the end of the resistance gene, there is over 98% sequence homology with the corresponding region of *ermC* from pE194. The alignment of the *ermM* DNA sequence with the *ermC* DNA sequence (Fig. 2) showed that the deletion begins right at the start of the 19-amino-acid leader peptide of the promoter region. The deletion begins 7 bp downstream from the first Shine-Dalgarno sequence (SD-1) at the first codon (ATG) of the peptide and extends beyond the end of the leader peptide to within 3 bp of the second Shine-Dalgarno sequence (SD-2) (29). The Pribnow box or "−10" sequence, the potential RNA polymerase recognition site or "−35" sequence, and the putative start of transcription (+1) are indicated in Fig. 2. These sequences are so designated because of their conformity to consensus sequences for bacterial promoters (27) and because they are identical to the sequences identified for the *ermC* determinant from pE194 (5, 10). The Shine-Dalgarno sequences SD-1 and SD-2 are the putative ribosome-binding sites for the leader peptide and the 29,000-molecular-weight methylase (29K methylase), respectively, based on their strong complementarity to the 3′ terminus of 16S rRNA from *Bacillus subtilis* (21, 29). Similarly, a complementary inverted repeat sequence at the 3′ end of the methylase gene is designated as the possible transcription terminator and is identical to one of the sequences proposed for *ermC* (5, 12).

Methylase gene. The probable ATG start codon for the 29K methylase protein of *ermM* is the same as that in *ermC* and is indicated in the sequence (Fig. 2). Because of the 107-bp deletion, it is now preceded by two tandem ribosome-binding sites, SD-1 and SD-2, which are separated by only 10 bp. The 29K methylase of *ermM* of pNE131 has the same number (244) of codons and amino acids as the methylase of *ermC* of pE194 and is thus presumed to encode a protein of similar molecular weight. The sequence of the methylase of *ermM* differs from that of the methylase of *ermC* at only 7 bases, resulting in seven different codons and coding for only five different amino acids. The codon usage for *ermM*, as has been noted for *ermC* (9), complies with the low guanine-plus-cytosine base composition for staphylococcal genes (32% for methylase codons) with a preference for A or U at the third base position (19). Among the seven codons that differ between the *S. epidermidis* gene and *ermC*, only two differ in the third ("wobble") base, and they do not encode a different amino acid.

DISCUSSION

From sequence data of the 5′ promoter region of the *ermM* gene of pNE131, a deletion was discovered which closely resembles that in *ermC* deletion mutants selected by growth

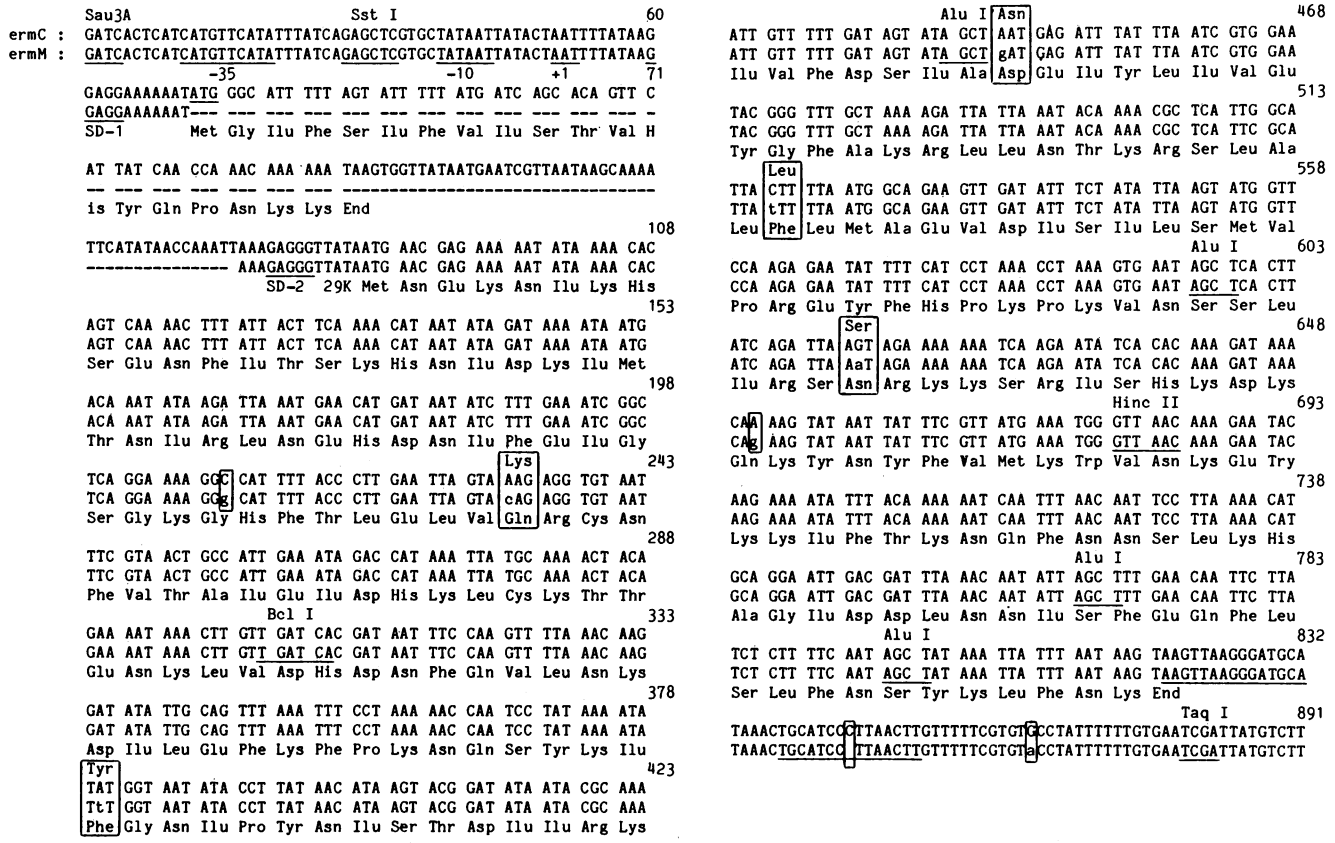

FIG. 2. Nucleotide sequence of *ermM* from *S. epidermidis*(pNE131) aligned with the homologous sequence of *ermC* from *S. aureus*(pE194). The positions of the *ermM* promoter (−35 and −10) are indicated by underlining, as are the possible start of transcription (+1) and ribosome-binding sites SD-1 and SD-2. Relevant restriction endonuclease recognition sites and the probable transcription terminator (bp 817 to 852) are also identified. The dashes indicate the deleted sequence of *ermM*. Boxes highlight a different nucleotide base or a codon triplet which designates a different amino acid. A different amino acid in the methylase of *ermC* relative to *ermM* is shown above a codon base triplet.

on tylosin plates (11) or generated in vitro by Bal 31 exonuclease digestion (8). An analysis of mutations to constitutive expression of resistance has been used to test the induction model for *ermC* (5, 10). The model for the inducible expression of *ermC* by use of a subinhibitory concentration of erythromycin proposes that a 141-bp leader region at the 5′ end of the *ermC* mRNA is capable of folding into a secondary hairpin structure (20). This structure forms a translational attenuator by sequestering the ribosome-binding site, SD-2, and the initiation codon, AUG, for the 29K methylase, thus preventing full expression of MLS resistance (Fig. 3a). In addition to its potential secondary structure, the 5′-controlling region contains a small open reading frame capable of encoding a 19-amino-acid peptide. It is believed that induction to full expression occurs when erythromycin-sensitive ribosomes, in the process of translating the small leader peptide, become stalled, owing to the presence of a small amount of erythromycin (3). When ribosomes bound to the antibiotic stall in this region of the message, they precipitate a change in the secondary structure of the mRNA to an active conformation in which the initiation signals SD-2 and AUG become unpaired (Fig. 3b). The mRNA is now accessible to ribosomes which may be either unexposed to or resistant to erythromycin and which are capable of initiating translation of the 29K methylase gene (7, 11).

Several mechanisms which result in constitutive expression have been found to occur among mutants selected in the laboratory. For example, a point mutation can occur in complementary repeat sequence 3 (Fig. 3a), disrupting its ability to form a stem-loop structure with repeat sequence 4, thus freeing SD-2, the ribosome-binding site for the translation of erythromycin methylase (5, 10). Direct tandem duplication of repeat segments 3 and 4, which ensure that one set of initiation signals is always unpaired for expression, has also been found (5). A third type of constitutive mutant involves deletions of the leader peptide region, similar to the deletion found in *ermM*.

A comparison of the leader region sequences from *ermC* and *ermM* reveal that the 107-bp deletion in the *S. epidermidis* determinant removes the 19-amino-acid leader peptide and extends to within 3 bp of SD-2, the ribosome-binding site for the 29K methylase peptide. The consequences of this deletion are more apparent when the 5′ leader regions of the corresponding mRNAs are compared. Figure 3a shows the message of *ermC* folded into one of the potential inactive conformational hairpin structures described by Horinouchi and Weisblum (11). The deletion in *ermM* not only excises the leader peptide but also removes complementary inverted repeat segments 1, 2, and 3 (Fig. 3b). This deletion eliminates all the complementary sequences able to pair up and sequester the translational initiation signals of the 29K methylase, resulting in constitutive expression of the *S. epidermidis ermM* gene. The extent of the deletion through segment 3 is significant for high-level constitutive expression. An analysis of deletions in the *ermC* leader region by

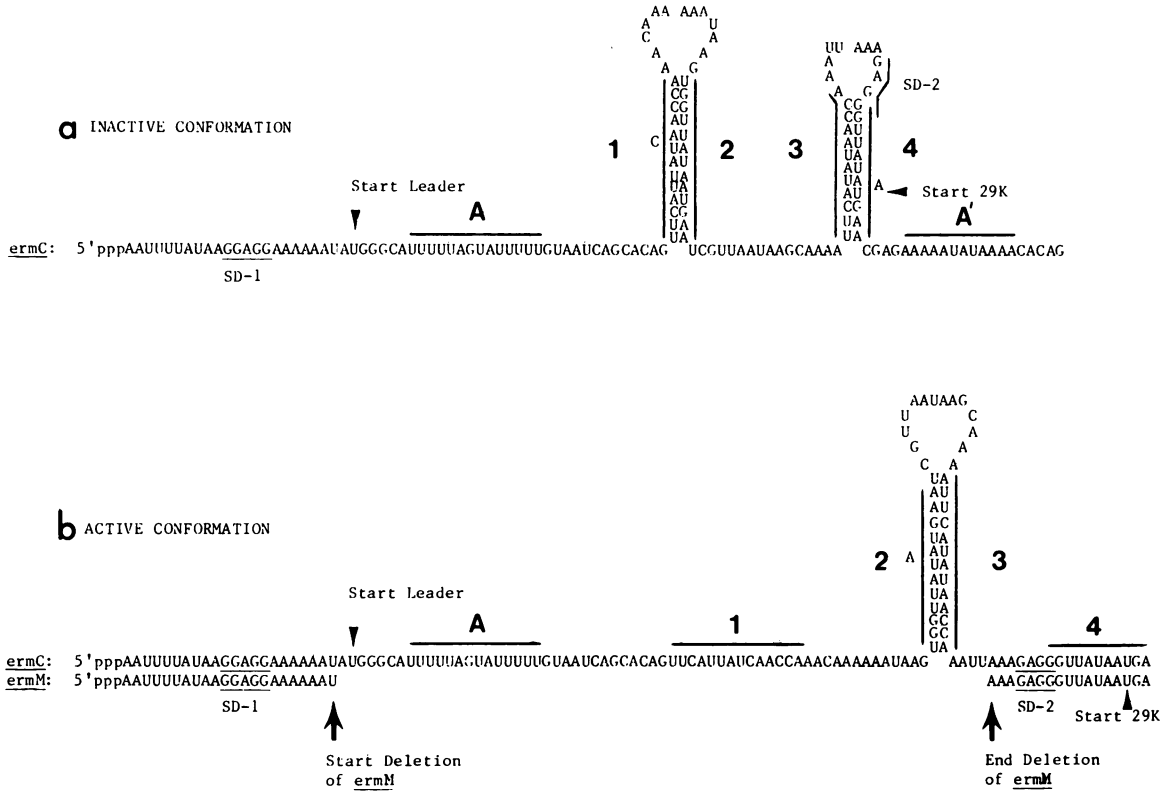

FIG. 3. Sequence of bases of the *ermC* and *ermM* mRNA transcripts deduced from the DNA sequences. Secondary stem-loop structures postulated by Horinouchi and Weisblum (11) to form in the mRNA encoding the methylase protein are shown. Panel a shows one possible inactive conformation of the *ermC* mRNA in which ribosome-binding site SD-2 and the AUG initiation codon for the 29K methylase are sequestered, preventing expression. Panel b shows the change in the *ermC* regulatory region to the active conformation following induction by erythromycin. In addition, the sequence of *ermM* mRNA is paired with that of *ermC* mRNA to show the extent of the deletion which removes sequences able to form stem-loop structures. Complementary repeat sequences are designated A, 1, 2, 3, 4, and A' (heavy lines above the sequences) as described by Horinouchi and Weisblum (11).

Hahn et al. (8) showed that deletions which extend only into segment A of the leader peptide or into segment 2 result in low-level expression of MLS resistance. Although induction is abolished by these deletions, full expression of MLS resistance is apparently hampered because alternative inactive mRNA structures can form. Only deletions which extend through complementary sequence 3 (or apparently into segment 1) result in high-level constitutive resistance like the phenotype encoded by *ermM*.

Recent studies have noted a predominance in the number of clinical isolates of staphylococci which display constitutive MLS resistance as opposed to the inducible phenotype (34; W. D. Jenssen, S. Thakker-Varia, D. T. Dubin, and M. P. Weinsein, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, A8, p. 2). Our studies with staphylococcal isolates, particularly *S. epidermidis*, have revealed a similar preponderance of the constitutive phenotype (B. Lampson and J. T. Parisi, Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, H164, p. 118). In addition, other studies suggest that there may have been a trend over the last 20 years toward an increasing proportion of *S. aureus* strains which express MLS resistance constitutively (4, 22). One may speculate that the predominant occurrence of the constitutive phenotype may be the result of the increasing clinical use, over the last 20 years, of the lincosamide clindamycin. In addition, the use of other MLS antibiotics, such as tylosin in veterinary medicine, may also provide the natural selective force for this trend. As already noted, investigators frequently obtain mutations to constitutive expression by growing an inducible strain of *S. aureus* (5, 11) or *B. licheniformis* (6) in the presence of the noninducer macrolide tylosin. It is perhaps not unreasonable to speculate further that the selection (and spread) of *S. epidermidis* strains containing a plasmid like pNE131 may have occurred in a similar fashion. Indeed, an analysis of plasmids contained in several constitutive *S. epidermidis* strains showed that the majority (75%) were indistinguishable from pNE131 (Lampson and Parisi, Abstr. Annu. Meet. Amer. Soc. Microbiol. 1984).

The *ermM* determinant from plasmid pNE131 shares a nearly identical nucleotide sequence with the *ermC* determinant from plasmid pE194. The sequence of pNE131 remains closely homologous for about 220 bp 5′ to the start of transcription of the methylase gene, at which point the sequence of pNE131 sharply diverges from that of pE194. Likewise, about 80 bp downstream from the *ermM* termination codon the sequence of pNE131 differs markedly (3′ from the *Hha*I site) from the homologous region of pE194. Other regions of pNE131 which have been sequenced thus far do not show any close homology with those of pE194 (unpublished data). Clearly, there has been a transfer of the *ermC* class of MLS resistance genes between the two species of staphylococci. Whether *ermC* is the ancestral gene of *ermM* is currently being investigated. By comparing the complete nucleotide sequences of both plasmids, we may be able to

discern more clearly the ancestral relationship between these two erythromycin resistance genes.

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