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Characterization of a Macrolide, Lincosamide, and Streptogramin Resistance Plasmid in *Staphylococcus epidermidis*

JOSEPH T. PARISI,[1]* JEFF ROBBINS,[2] BERT C. LAMPSON,[1] AND DAVID W. HECHT[1]

Departments of Microbiology[1] and Biochemistry,[2] School of Medicine, University of Missouri, Columbia, Missouri 65212

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A strain of *Staphylococcus epidermidis* was transduced to erythromycin resistance, and all of the transductants exhibited the macrolide, lincosamide, streptogramin B resistance phenotype. Curing and antibiotic disk studies also indicated that these resistances were controlled by a single plasmid determinant and were constitutive. Agarose gel electrophoresis of plasmid deoxyribonucleic acid (DNA) from donor, cured, and transduced strains showed that a single plasmid was responsible. This plasmid, designated pNE131, was examined for sequence homology to two other plasmids, pE194 and pI258, from *Staphylococcus aureus*, which also code for erythromycin resistance. DNA from plasmids pNE131 and pE194 hybridized with one another, but no extensive homology to pI258 with either pNE131 or pE194 was found. Restriction endonuclease digests of pNE131 and pE194 showed no common fragments. However, sequence homology was localized to the nucleotides in pE194 that code for the 29,000-dalton protein responsible for erythromycin resistance. pNE131 was calculated to have 2,220 base pairs and is the smallest naturally occurring plasmid with a known function yet reported in *S. epidermidis*.

Staphylococcus epidermidis is a potential pathogen, and its resistance to antibiotics can present therapeutic problems. The presence of resistance plasmids in this organism and recent reports of the interspecies transfer of these plasmids to *Staphylococcus aureus* have sparked new interest in this organism (9, 28). Evidence for the transfer of resistance plasmids from the ubiquitous and relatively avirulent, more antibiotic-resistant *S. epidermidis* to the more virulent *S. aureus* now exists, although whether this transfer among staphylococci in nature occurs by transduction or conjugation, or both, remains to be elucidated.

Recently, Weisblum et al. (27) characterized from an erythromycin-resistant strain of *S. aureus* a plasmid (pE194) with a molecular weight of 2.3×10^6 (3,727 base pairs [bp]). This plasmid confers an inducible resistance to the macrolide, lincosamide, and streptogramin B (MLS) antibiotics. Another plasmid (pI258) from *S. aureus* conferring constitutive resistance to erythromycin has also been characterized by Novick et al. (18); in addition, this plasmid codes for beta-lactamase production and heavy metal resistance and has a molecular weight of 18.8×10^6. In this study, we describe the transduction in clinical isolates of *S. epidermidis* of a plasmid, designated pNE131, which confers constitutive resistance to the MLS antibiotics. This plasmid's size was determined by analysis in both polyacrylamide and agarose gels, and its sequence homology to both pE194 and pI258 was explored.

MATERIALS AND METHODS

Bacterial strains and phages. *S. epidermidis* 750 was a clinical isolate obtained from Brooks Air Force Base in Texas. It was lysogenic and served as the donor strain in transduction. *S. epidermidis* 131 was a clinical isolate obtained from the University of Missouri Health Sciences Center in Columbia and served as the recipient. Phage typing was performed with the 11 phages in our collection (23), phages 407-1, 407-2, and 202 from L. E. Blouse (Brooks Air Force Base, Tex.), and phages Ph9, Ph10, and U14 from G. Pulverer (University of Cologne, Cologne, West Germany). *S. aureus* strain RN2442 containing pE194 and strain RN453 containing pI258 were obtained from B. Weisblum (University of Wisconsin, Madison). *Escherichia coli* V-517, containing eight well-characterized plasmids (13), was obtained from R. L. Warren (Wright State University, Dayton, Ohio). Biotype determination and production of urease, gelatinase, caseinase, lysozyme, phosphatase, DNase, hemolysins, and lipolytic activity by the transductants and recipient strains were as described previously (14). Antibiotic susceptibility to 12 antibiotics was determined

by the disk diffusion method (16). The macro broth dilution method (26) was used to determine the minimal inhibitory concentration (MIC) of transductants and recipient strains to erythromycin, oleandomycin phosphate, and tylosin tartrate (Sigma Chemical Co., St. Louis, Mo.), clindamycin, and ostreogrycin B, a streptogramin B-type antibiotic (a gift from B. Weisblum). Determination of resistance to heavy metals (cadmium, copper, zinc, arsenite, lead, and mercury) was according to Novick and Roth (19).

Transduction procedure. Induction of donor strain 750 with mitomycin C with the liberation of transducing phages was the same as described previously (22). The transduction procedure was the same as reported by Olson et al. (20) except that the medium used for the selection of resistant transductants contained 5 μg of erythromycin per ml. Loss of resistance to antibiotics upon exposure to sodium dodecyl sulfate (SDS) has been described (20) except that the SDS-exposed cells were replicated onto medium containing 5 μg of erythromycin per ml.

Preparation of DNA. The lysis of cells and preparation of a cleared lysate was by published methods (6, 21). When further purification of the plasmid DNA was required, the preparation was centrifuged through cesium chloride-ethidium bromide gradients, and the band corresponding to the plasmid DNA was collected and purified (2).

Restriction enzyme digestion. All enzymes were purchased from Bethesda Research Laboratories (Gaithersburg, Md.). The digestions were performed under their recommended conditions, except that a fourfold excess of enzyme was added.

Gel electrophoresis. The electrophoresis (E) buffer consisted of 40 mM Tris, 5 mM sodium acetate, and 1 mM EDTA (pH 7.9). Before use, ethidium bromide was added to a final concentration of 0.5 μg/ml. Agarose (Sigma, type II) was dissolved to a final concentration of 0.8% in E buffer containing ethidium bromide and was added to a horizontal slab apparatus (16 by 14 cm). Samples were electrophoresed in E buffer at 52 mA for 2.5 h. Electrophoresis in 5% polyacrylamide gels, made up in Tris-borate-EDTA buffer (15), was carried out at 30 mA for 2 h, and the samples were stained in this buffer containing ethidium bromide to a final concentration of 0.5 μg/ml. After electrophoresis, DNA bands were visualized by UV light and photographed with a Polaroid MP-4 camera with a UV barrier filter plus a no. 6 red filter and type 52 Polapan Polaroid film.

Nick translation of plasmid DNA. Plasmid DNA was nick-translated under the conditions of Maniatis et al. (15) with some modifications. The concentration of the three unlabeled substrates was 15 μM; 5 μl of the labeled deoxynucleotide triphosphate (with a specific activity of greater than 800 Ci/mmol at 10 mCi/ml) was added directly, bringing the reaction mixture to a final volume of 15 μl. The reaction was carried out at 15°C for 1 to 3 h, after which SDS was added to a final concentration of 0.1%. The reaction mixture was extracted with an equal volume of phenol-chloroform (1:1), and the high-molecular-weight products were separated from the unincorporated substrates by passage through a column (10 by 0.5 cm) containing Sephadex G-100 eluted with 0.01 M Tris, pH 7.0.

Transfer of DNA to DBM paper and hybridization. DNA electrophoresed in horizontal agarose slab gels was nicked with dilute HCl and rapidly transferred onto diazobenzyloxymethyl (DBM) paper as described by Wahl et al. (25). Prehybridization washes were performed in 2× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate) for 1 h at 65°C, followed by a 1- to 3-h wash at 65°C in 2.5× SSC, 5× Denhardt solution (3), 0.1% SDS, and 50 μg of denatured, low-molecular-weight salmon sperm DNA per ml. A 10-ml amount of the mixture was made 10% in dextran sulfate (25), and 3×10^6 dpm of the appropriate denatured probe was added. The hybridization proceeded at 65°C for 2 to 5 h with gentle shaking in a sealed plastic bag. The post-hybridization rinses consisted of five washes (5 min each) in 1× SSC at 65°C. The dried blots were autoradiographed with preflashed Kodak XRP-5 film for 5 h at −80°C, using two intensifying screens.

RESULTS

Transduction of MLS resistance. The donor and recipient strains and their derivatives were all biotype 1. The donor strain and its derivatives were lysed by phages 202, Ph10, and U14. The recipient strain and its derivative were lysed by phages 112 and 202. Donor strain 750 was resistant to penicillin (Pc), ampicillin (Am), kanamycin (Km), gentamicin (Gm), tetracycline (Tc), erythromycin (Em), oleandomycin (Om), tylosin (Ty), clindamycin (Cc), and streptogramin B (St). Of the antibiotics tested, it was sensitive to only chloramphenicol (Cm) and vancomycin (Va). Strain 750c was an isolate from a curing experiment, derived from strain 750, that was Em^s Om^s Ty^s Cc^s St^s. Strain 750ct was an Em^r transductant of 750c that was also Om^r Ty^r Cc^r and St^r. Transduction occurred at the frequency of 6×10^{-8} transductants per plaque-forming unit. Except for resistance to penicillin and ampicillin, recipient 131 was sensitive to the remaining 10 antibiotics tested. Upon transduction to resistance to erythromycin at a frequency of 1.4×10^{-7} transductants per plaque-forming unit, it also became Om^r Ty^r Cc^r St^r. The MICs of the donor, recipient, cured, and transduced strains are shown in Table 1. Between the transduced and recipient strains, there was at least a sixfold difference in the MIC of erythromycin, oleandomycin, tylosin, clindamycin, and streptogramin B. The method of Weisblum et al. (27), with erythromycin susceptibility disks placed in oppostion to disks containing other macrolides, lincosamides, or streptogramin B antibiotics on plates inoculated with the *S. epidermidis* donor strain and transductants, showed that the resistance to the MLS antibiotics was constitutive.

To determine whether genetic markers other than those for resistance to the MLS antibiotics were transduced jointly, the transduced and re-

TABLE 1. *MICs of donor, recipient, cured, and transduced strains of S. epidermidis*

Strain	MIC (μg/ml)[a]				
	Em	Om	Ty	Cc	St
750	64	64	64	64	64
750c[b]	0.125	1.0	0.25	0.125	8
750ct[c]	64	64	64	64	64
131	0.125	0.5	0.5	0.125	8
131t[d]	64	64	64	64	64

[a] Em, Erythromycin; Om, oleandomycin; Ty, tylosin; Cc, clindamycin; St, streptogramin B.
[b] Cured isolate of donor 750.
[c] Transductant of 750c.
[d] Transductant of recipient 131.

cipient strains were characterized more fully. No change was noted in urease, gelatinase, caseinase, lysozyme, phosphatase, DNase, hemolysins, and lipolytic activity. Resistance to all of the heavy metals tested remained unchanged for transduced and recipient strains.

Resistance to erythromycin was lost from donor strain 750 upon growth in 30 μg of sodium dodecyl sulfate per ml at the rate of 39% of 934 colonies screened. Cells from 10% of the cured colonies were tested and were identical in both phage type and antibiotic susceptibility; these had also lost resistance to oleandomycin, tylosin, clindamycin, and streptogramin B. When cured isolates were tested for changes in biochemical activities and loss of heavy metal resistance, none was different from donor strain 750.

Plasmid isolation and characterization. The plasmid DNA of *S. epidermidis* donor strain 750, its cured derivative 750c, and transduced strains 750ct and 131t was examined by agarose gel electrophoresis (Fig. 1). Lane 1 contains the DNA of donor strain 750 with four prominent bands and a heavy broad band of chromosomal DNA which was not removed in the extraction procedure. Lane 2 contains the DNA of 750c, cured of resistance to erythromycin. The band at the bottom of the gel present in lane 1 is missing in lane 2 and represents the plasmid DNA responsible for MLS resistance. The band above the MLS plasmid band is the tetracycline plasmid. Curing studies have resulted in the loss of this plasmid band from strain 750. It has been transduced back into 750c (unpublished data), and its position in the gel is in agreement with our earlier published report of this plasmid (20). The other bands are cryptic plasmids. Lane 3 with the DNA of a transductant of 750c, resistant to the MLS antibiotics, has the bottom band which was present in donor strain 750 (lane 1). Lane 4 with the DNA of the recipient 131 before transduction had only a chromosomal band, but the DNA of a transductant of 131, resistant to the MLS antibiotics, gained a single plasmid band, corresponding to the MLS plasmid band in donor strain 750 (lane 5). Lane 6 contains the plasmid DNA from *E. coli* V-517. It contained seven plasmid species (arrows) ranging in size from 2,100 to 7,200 bp used as reference molecules (13). The other bands seen in the gel are multiple forms of these seven plasmids and were not used as reference molecules.

Homology of plasmid pNE131 with other MLS plasmids. We determined the extent of homology between pNE131 from *S. epidermidis* and pE194 or pI258 from *S. aureus* (Fig. 2). The nick-translated DNA of pI258 demonstrated no homology with either pNE131 or pE194 (Fig. 2A). The DNA of pNE131 hybridized strongly with that of pE194 (Fig. 2B). Figure 2C confirmed the results of the preceding two panels; nick-translated DNA of pE194 hybridized strongly with DNA derived from pNE131 only. Thus, pI258 contained no extensive homologies with either pE194 or pNE131, whereas the latter two plasmids contained closely related sequences.

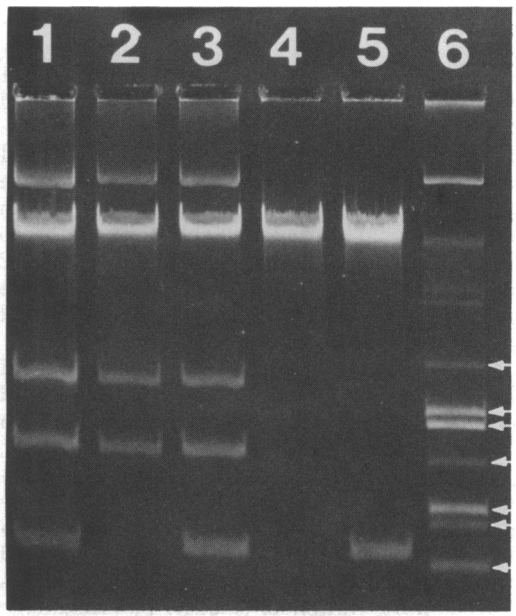

FIG. 1. *Agarose gel electrophoresis of plasmid DNA from S. epidermidis strains 750, 131, and derivatives. (1) 750 (Tc[r] Em[r]); (2) 750c (cured of erythromycin resistance, Tc[r]); (3) 750ct (Tc[r] Em[r] transductant); (4) 131 (Tc[s] Em[s]); (5) 131t (Tc[s] Em[r] transductant); (6) E. coli V-517 plasmids as markers. The sizes (base pairs) of the seven plasmids (arrows), beginning at the bottom, are 2,100, 2,700, 3,000, 3,900, 5,100, 5,550, and 7,200. The broad band in lanes 1 to 5 is chromosomal DNA.*

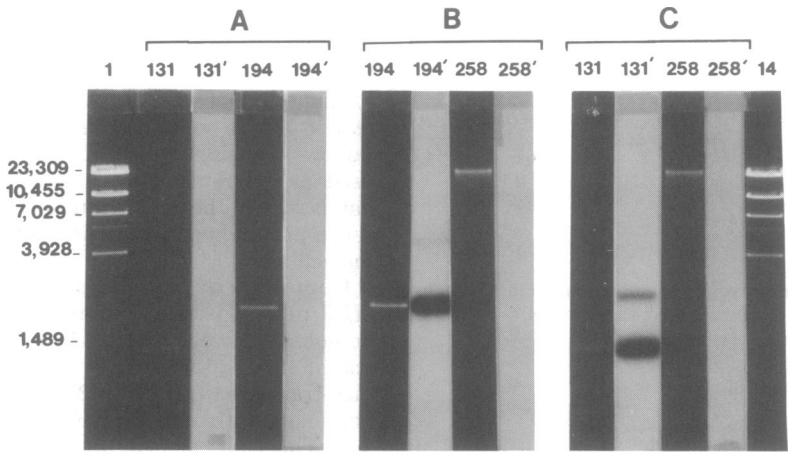

FIG. 2. *DNA-DNA hybridization with MLS resistance plasmids. The nick-translation-labeled probe used in each of the hybridizations was: (A) pI258, (B) pNE131, and (C) pE194. Lanes 1 and 14 are BamHI digestions of λ Charon phage 4A. The fragment lengths are indicated in base pairs. The remaining lanes alternate, displaying the plasmid DNA electrophoresed on agarose gels and the corresponding autoradiograph. The faint bands in (B) and (C), designated 194' and 131', result from trace amounts of non-supercoiled plasmid DNA.*

Restriction enzyme digests of plasmid pNE131 and pE194 DNAs. Restriction digests of plasmids pNE131 and pE194 were made, and the products were analyzed in 5% polyacrylamide gels. Figure 3 shows *Hin*fI and *Taq*I digests of the two plasmids. Digestion of pNE131 with *Hin*fI yielded two fragments of 2,000 and 220 bp, neither of which was present in pE194. Digestion of the two plasmid DNAs with *Taq*I also resulted in two unique patterns, with pNE131 containing a 1,400-bp fragment and an additional 820-bp fragment that migrated close to but slightly faster than *Taq*I fragment C (932 bp) derived from pE194. A detailed restriction map of pE194 with the sizes of the *Hin*fI and *Taq*I fragments has been published (7).

Hybridization of radioactive plasmid DNA to restriction digests of pE194 and pNE131. A preparative digestion of pE194 with *Taq*I resulting in three (*Taq*I-A [1,442 bp], *Taq*I-B [1,353 bp], and *Taq*I-C [932 bp]) fragments was separated and isolated from agarose gels. Each of these fragments was then nick-translated and hybridized with the *Hin*fI and the *Taq*I digests of pNE131 that had been transferred onto DBM paper (Fig. 4). Two fragments of 2,000 and 220 bp resulted from the *Hin*fI digest of pNE131, and *Taq*I digestion yielded two fragments of 1,400 and 820 bp. The *Taq*I-A region of pE194 hybridized strongly with the longer *Hin*fI fragment (Fig. 4, lane 1). No hybridization to the 220-bp fragment was observed. In contrast, both *Taq*I fragments derived from pNE131 hybridized with the probe. No hybridization was observed with identical digests when *Taq*I-B or *Taq*I-C were used as probes (data not shown). From these studies we infer that pNE131 and pE194 contain homologous sequences demonstrable by DNA-DNA hybridization, localizable to pE194 *Taq*I fragment A, which contains the MLS resistance determinant.

DISCUSSION

Transductional analysis and curing studies have shown that erythromycin resistance in *S. epidermis* 750 is controlled by a plasmid which also confers resistance to the MLS antibiotics. Plasmid pE194, which has been characterized in *S. aureus*, confers the MLS resistance phenotype, and this resistance is inducible (27). Some streptococci also exhibit the MLS resistance phenotype, and it has been shown by DNA sequence homology that some streptococcal MLS resistance plasmids and the *S. aureus* MLS resistance plasmid pI258 share a small common segment of 3,000 to 4,500 bp (4). Because of the apparent widespread distribution of the MLS resistance phenotype, a transposon mechanism was suggested (17). Recently, the erythromycin resistance determinant in *S. aureus* plasmid pI258 has been reported to reside on a 5.3-kilobase transposon, Tn*551*, which is structurally similar to Tn*3* in *E. coli* (10).

Antibiotic resistance plasmids have been transferred in *S. aureus* by transformation (12, 24) and by transduction in both *S. aureus* (8, 18) and *S. epidermidis* (6, 20) and interspecifically by mixed cultivation in vitro or on human skin (9, 28). The mechanism of the mixed-culture transfer remains to be elucidated since lysogeny

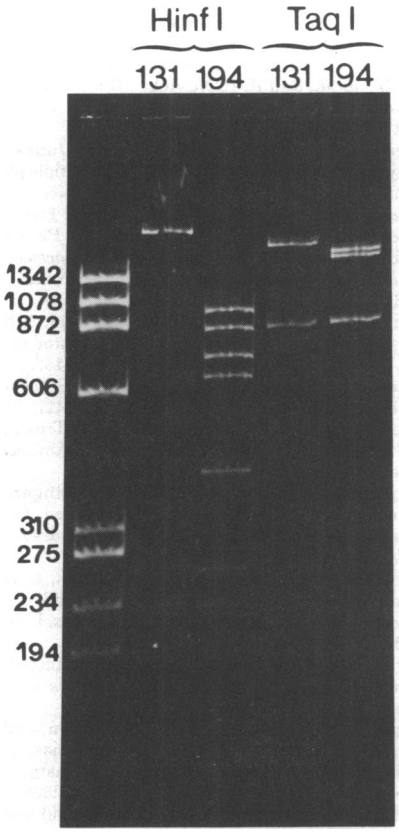

FIG. 3. *HinfI and TaqI restriction endonuclease digestions of pNE131 and pE194 DNA. A HaeIII digest of phage φ174 at the extreme left was used as a marker for determination of the molecular weight. The sizes of the HaeIII fragments are indicated in base pairs.*

of the donor or recipient or both appears to be necessary and has been termed "phage-mediated conjugation" (11). In *S. aureus* and *S. epidermidis*, the isolation of tetracyline resistance plasmids, chloramphenicol resistance plasmids, and gentamicin resistance plasmids that appear identical by a variety of biochemical criteria supports the interspecies transfer of these plasmids in nature (5, 8, 9, 24), but the data obtained in our study argue against the identity of pNE131 and pE194. MLS plasmid pE194 in *S. aureus* is 3,727 bp in length (7). An accurate size estimate (2,220 bp) of our plasmid (pNE131) was obtained from the restriction fragments electrophoresed in both polyacrylamide and agarose gels. The hybridization data showed that pNE131 shares no extensive homology with pI258. However, it must be pointed out that small (3 to 15 bp) stretches of identical or related DNA would probably not be detected by the methods used. Thus, it would be premature to conclude that pNE131 (along with pE194) is totally divergent from pI258.

pE194 showed extensive homologies with the smaller plasmid, pNE131. Not surprisingly, this homology was localized in the *Taq*I-A region of pE194 (Fig. 4), which has been shown to code for a protein that confers erythromycin resistance and has a molecular weight of 29,000 (7). However, the two plasmids obviously do not share identical sequences in this region, as evidenced on a gross level by the different fragments generated by restriction endonuclease digestion (Fig. 3). Our unsuccessful attempt to find other properties of *S. epidermidis* which might be controlled by pNE131 is not surprising in view of its small size. Approximately 900 bp of DNA is necessary to code for the 29,000-dalton protein that we hypothesize pNE131 produces. This leaves approximately 1,320 bp of DNA that must be responsible for its maintenance and replication. The sequencing of the entire plasmid should yield information regard-

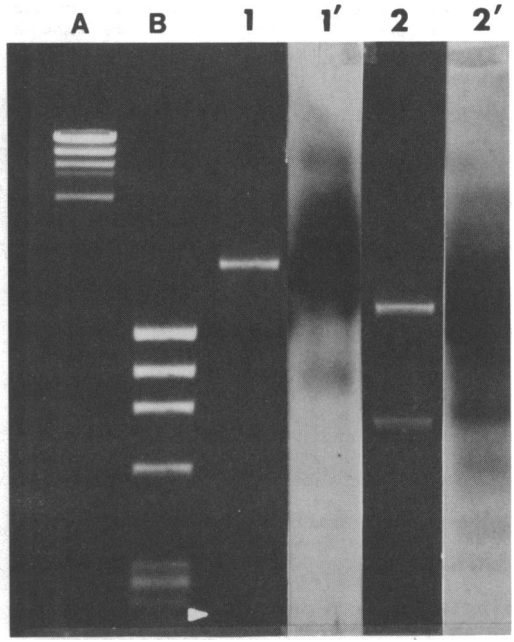

FIG. 4. *DNA-DNA hybridization of HinfI- or TaqI-digested pNE131. TaqI fragment A from pE194 was nick-translated to a specific activity of 75 Ci/mmol with [^{32}P]dCTP. (1) HinfI-digested pNE131; the white arrow denotes the position of the 220-bp fragment that cannot be seen in this photograph. (1') The corresponding autoradiograph. (2) TaqI-digested pNE131. (2') The corresponding autoradiograph. (A) BamHI digest of λ Charon phage 4A. (B) HaeIII digest of phage φX174. The fragment sizes of these markers are indicated in Fig. 2 and 3, respectively. Note that the autoradiographs were overexposed so that even weak signals would be detected.*

ing the exact nature of homology with pE194 and the nature of the other proteins encoded by the plasmid. This work is now in progress.

To our knowledge pNE131, which has been characterized in this study, is the smallest naturally occurring *S. epidermidis* plasmid whose function has been elucidated to date. When this manuscript was being prepared, a communication appeared (1) that described the isolation from *S. aureus* of a constitutive erythromycin resistance plasmid, designated RJ5, with a size of 2,100 bp. A comparison of pNE131 and pRJ5 would be desirable.

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