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Transposon-Mediated Amikacin Resistance in *Klebsiella pneumoniae*†

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A multiresistant *Klebsiella pneumoniae* strain isolated from neonates in Mendoza, Argentina, harbored a 48-kilobase-pair (kbp) plasmid, pMET1, with genetic determinants for resistance to amikacin and also ampicillin, kanamycin, streptomycin, and tobramycin. This plasmid was compared with pJHCMW1, a previously isolated 11-kbp plasmid carrying transposon Tn*1331*, which encodes resistance to amikacin, as well as ampicillin, kanamycin, streptomycin, and tobramycin, and which was originally present in a *K. pneumoniae* strain that caused an outbreak in a hospital in Buenos Aires, Argentina. The comparison demonstrated that the replication regions of the two plasmids are unrelated. However, in pMET1 an 11-kbp transposition element, Tn*1331.2*, was identified; it was closely related to Tn*1331*, with the difference that a 3-kbp *Bam*HI DNA fragment carrying the aminoglycoside resistance genes was duplicated in tandem.

Multiresistant *Klebsiella pneumoniae* strains are frequently the cause of outbreaks of hospital infection with a high mortality rate. About 6% of patients in the United States acquire bacterial infections while in hospitals (27). Aminoglycosides are very important weapons against these infections, but resistance is a growing clinical problem (7).

Usually, aminoglycoside resistance found in clinical isolates is due to the presence of modifying enzymes (1) which may be coded for by transposons (5). Among the aminoglycosides, amikacin (13) is the most refractory to inactivating enzymes (21, 24, 27). Although the incidence of resistance to amikacin has remained low, chromosome- and plasmid-borne resistance has been reported (11, 12, 16, 22, 33, 34). Genetic determinants encoding resistance to amikacin were found in two transposable elements, Tn*2424* (19) and Tn*1331* (30). This latter transposon also encodes resistance determinants for ampicillin, kanamycin, streptomycin, and tobramycin and was identified in an 11-kilobase-pair (kbp) plasmid, pJMCMW1. This plasmid was isolated from a *K. pneumoniae* strain causing meningitis in a neonate in a children's hospital in Buenos Aires, Argentina (34). The gene encoding amikacin resistance in Tn*1331* was recently cloned and sequenced (20, 31). The sequence was identical to that reported by Tran Van Nhieu and Collatz in a multiresistance plasmid (32). The gene encoding the acetyltransferase which modifies amikacin, as well as kanamycin and tobramycin, is followed by a structural gene for an adenylyltransferase which confers resistance to streptomycin in the same transcriptional unit (20, 32).

In this paper, we report the isolation and analysis of pMET1, a plasmid encoding amikacin resistance, harbored by a *K. pneumoniae* strain recently isolated from neonates in an outbreak of hospital infection that led to the deaths of several neonates in a pediatric unit. This plasmid harbors a Tn*1331*-related transposon, Tn*1331.2*, which has a duplication of the DNA fragment carrying the amikacin resistance gene.

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MATERIALS AND METHODS

Bacterial strains and plasmids. *K. pneumoniae* FC1 was isolated from diseased children in a pediatric unit in Mendoza, Argentina (Hospital Luis C. Lagomaggiore). The bacterial strains and plasmids used are described in Table 1.

Preparation and electrophoresis of plasmid DNA. Plasmid DNA was prepared by the method of Birnboim and Doly (2) as described previously (31). Electrophoresis of cleaved DNA was performed in a horizontal 0.7% (wt/vol) agarose gel as previously described (31).

Molecular cloning experiments and conjugations. Treatments with restriction endonucleases and T4 DNA ligase were performed as recommended by the supplier (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). Transformation of competent *Escherichia coli* HB101 cells was performed as described previously (29). Conjugations were performed on solid medium as described before (29), by using a donor-recipient ratio of 1:10. The frequency of transfer of pMET1 was determined as described by Curtiss (6).

Determination of compatibility of plasmids. Plasmid compatibility was studied essentially as described by Timmis et al. (28). Incompatibility was tested by transformation of pMET14 and pROX2 into *E. coli* HB101. A culture of *E. coli*(pMET14, pROX2) was then incubated for 16 h in the absence of selection, and the cells were analyzed for their plasmid content.

Southern blot hybridizations. Restriction fragments were transferred to nitrocellulose paper by the Southern blot transfer method (26). Hybridization experiments were performed under high-stringency conditions by placing the filters in bags containing 0.75 M NaCl, 0.075 M sodium citrate, Denhardt solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 1 mM EDTA, 0.1% sodium dodecyl sulfate, and 50% formamide. After 3 h at 37°C, heat-denatured ^{32}P-labeled DNA and 100 μg of carrier DNA per ml were added and the bags were incubated for 16 h at 37°C. The filters were washed at 65°C in a solution containing 0.3 M NaCl, 0.03 sodium citrate, and 0.1% sodium dodecyl sulfate. DNA was labeled by using a random primed DNA labeling kit as recommended by the

TABLE 1. Bacteria and plasmids

Strain or plasmid	Relevant genotype or phenotype[a]	Reference or source
Bacteria		
E. coli HB101	*pro leu thi lac hsr hsm endA recA rpsL20*	4
E. coli C2110	*polA rha his* Nal^r Rif^r	15
K. pneumoniae FC1	Ak^r Ap^r Cm^r Km^r Mz^r Nm^r Sm^r Tm^r	This work
Plasmids		
pBR325	Ap^r Cm^r Tc^r; pMB1 replicon	3
pJHC-A18	*cat* gene from pRI1234 cloned in pBR322	25
pJHCMW1	Ap^r Ak^r Km^r Sm^r Tm^r	34
pMET1	Ap^r Ak^r Km^r Sm^r Tm^r Tra$^+$ Mob$^+$ (Fig. 2)	This work
pMET14	Deletion derivative of pJHCMW1; Ak^r Km^r Tm^r	30
pPEM10	Deletion derivative of pMET1 with the *cat* gene from pJHC-A18; Ap^r Ak^r Cm^r Km^r Tm^r Tra$^-$ Mob$^+$ (Fig. 2)	This work
pRK2013	Km^r Tra$^+$ Mob$^+$	9
pROX1	3-kbp *Bam*HI pMET1 fragment carrying the Ak^r gene cloned in pBR325; Ak^r Ap^r Km^r Sm^r Tm^r	This work
pROX2	Deletion derivative of pMET1; Ap^r (Fig. 2)	This work
pROXT1	2.3-kbp *Eco*RI pJHCMW1 fragment carrying the replication region ligated to the Km^r fragment of pUC4K	This work
pUC4K	Ap^r Km^r; pMB1 replicon	Pharmacia
pVK102	Tc^r Km^r; RK2 replicon; Tra$^-$ Mob$^+$	14

[a] Ap^r, Ampicillin resistance; Ak^r, amikacin resistance; Cm^r, chloramphenicol resistance; Km^r, kanamycin resistance; Mz^r, mezlocillin resistance; Nal^r, nalidixic acid resistance; Nm^r, netilmicin resistance; Rif^r, rifampin resistance; Sm^r, streptomycin resistance; Tc^r, tetracycline resistance; Tm^r, tobramycin resistance.

supplier (Boehringer Mannheim Biochemicals, Indianapolis, Ind.).

Antibiotic susceptibility. Antibiotic susceptibility was determined by a standard single-disk method as previously described (34).

Determination of aminoglycoside *N*-acetyltransferase. *N*-acetyltransferase activity was determined by the phosphocellulose paper binding assay (10).

RESULTS

Isolation of pMET1 and molecular cloning of amikacin resistance determinants. A *K. pneumoniae* strain was the causative agent of a nosocomial outbreak which occurred in a pediatric unit of a hospital in Mendoza, Argentina, in May 1986. This strain was resistant to amikacin, ampicillin, carbenicillin, chloramphenicol, kanamycin, mezclocillin, netilmicin, streptomycin, tetracycline, and tobramycin. Analysis of the plasmid content of this strain revealed the presence of a 48-kbp plasmid called pMET1 which encoded resistance to amikacin, ampicillin, kanamycin, streptomycin, and tobramycin. Conjugations using the original *K. pneumoniae* FC1 as the donor and *E. coli* C2110 as the recipient with amikacin and nalidixic acid for selection led to the isolation of *E. coli* C2110 harboring pMET1. This strain, *E. coli* C2110(pMET1), was then mated with *E. coli* HB101. Selection in the presence of amikacin, in addition to inability to use lactose, allowed the isolation of *E. coli* HB101(pMET1), indicating that this plasmid is conjugative. The frequency of transfer of pMET1 was >1 for conjugation times of 3 and 8 h, suggesting that this plasmid is derepressed for transfer, as happens with some R factors (8). The pattern of antibiotic resistances conferred by pMET1 was identical to that present in a strain carrying pJHCMW1, an 11-kbp plasmid recently isolated from another multiresistant *K. pneumoniae* strain (34). Digestion of both plasmids with *Bam*HI resulted in production of a 3-kbp DNA fragment (Fig. 1a). In pJHCMW1, it is known that this 3-kbp DNA fragment carries the gene encoding resistance to amikacin. To examine whether the pMET1 3-kbp *Bam*HI fragment was homologous to that from pJHCMW1, Southern blot hybridization of the gel shown in Fig. 1a was performed, using the 3-kbp *Bam*HI DNA fragment from pJHCMW1 as a probe. The result of this experiment showed that the 3-kbp *Bam*HI DNA fragment generated by digestion of pMET1 is homologous to the probe (Fig. 1b).

To clone the genetic determinants encoding amikacin resistance, pMET1 DNA was digested with *Bam*HI and ligated with *Bam*HI-digested pBR325. The recombinant clone, pROX1 (Fig. 2), carried a 3-kbp DNA fragment encoding resistance to amikacin. Restriction endonuclease analysis and comparison of pROX1 with pJHC-T00181, which is a recombinant plasmid obtained by cloning the 3-kbp *Bam*HI DNA fragment carrying the genetic determinants for amikacin resistance from pJHCMW1 (31), demon-

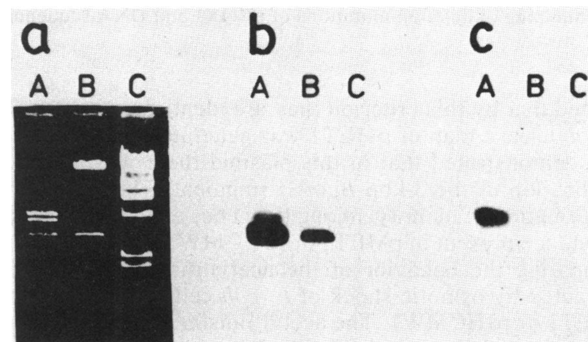

FIG. 1. (a) Agarose gel electrophoresis of *Bam*HI-digested pJHCMW1 (lane A) and pMET1 (lane B). (b) Southern blot hybridization of the gel, using as a probe the radioactively labeled 3-kbp *Bam*HI DNA fragment from pJHCMW1. (c) Southern blot hybridization of a gel similar to that shown in panel a, using as a probe the replication region of pJHCMW1. Lanes C contained *Hin*dIII-digested lambda DNA.

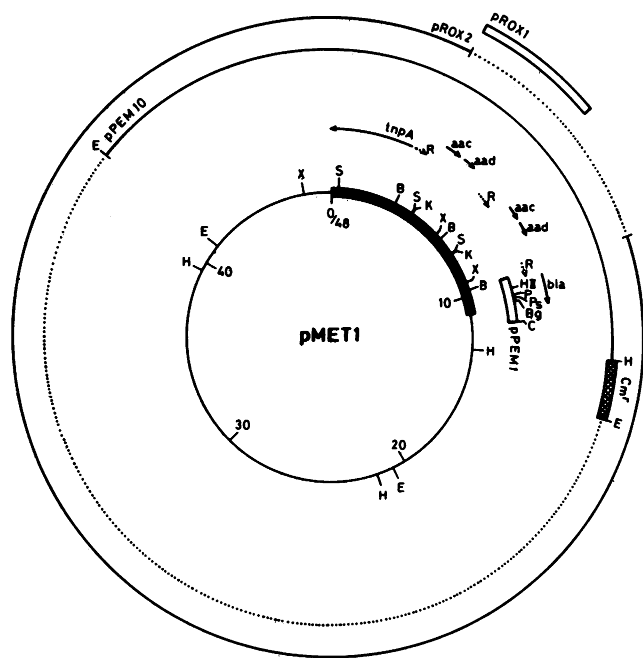

FIG. 2. Genetic and physical maps of pMET1. The diagram shows cleavage sites for the following restriction enzymes: *Bam*HI (B), *Bgl*I (Bg), *Cla*I (C), *Eco*RI (E), *Hin*dII (HII), *Hin*dIII (H), *Kpn*I (K), *Pst*I (Ps), *Pvu*I (P), *Sst*I (S), and *Xho*I (X). The numbers indicate coordinates in kilobase pairs. The heavy bar on the pMET1 map represents Tn*1331.2* DNA. Dotted lines indicate deleted DNA fragments. Plasmid pPEM10 was generated by deletion of the *Hin*dIII-*Eco*RI fragment shown and insertion of the pJHC-A18 DNA fragment carrying the chloramphenicol resistance gene (cross-hatched bar). Open bars represent cloned pMET1 DNA fragments. Plasmid pROX1 was generated by cloning of the 3-kbp *Bam*HI fragment with pBR325 as the cloning vector. Plasmid pPEM1 was obtained by cloning of a *Bam*HI-*Cla*I pMET1 fragment carrying the gene for resistance to ampicillin in a derivative of pBR325 lacking the *bla* gene. Plasmid pROX2 was generated by deletion of both 3-kbp *Bam*HI fragments. The dotted arrows (R) indicate regions with homology to the Tn*3* *tnpR* gene, as determined by hybridization by using the 0.3-kbp *Hae*II-*Bam*HI fragment from Tn*3*, which carries most of the *tnpR* gene, as a probe. The diagram also indicates the location of the *tnpA* gene. aac indicates the location of the gene encoding the 6'-*N*-acetyltransferase that modifies amikacin, as determined by restriction mapping of deletion and insertion mutations of pROX1. aad indicates the location of the adenylyltransferase gene which confers resistance to streptomycin, as determined by restriction mapping of deletion mutations of pROX1 and DNA sequencing (20).

strated that by this criterion they are identical. A restriction endonuclease map of pMET1 was generated, and this analysis demonstrated that in this plasmid there was a tandem duplication of the 3-kbp *Bam*HI fragment (Fig. 2). Further confirmation of identity among the genes encoding amikacin resistance present in pMET1 or pJHCMW1 was achieved by comparing the behavior of the acetyltransferase activities extracted by osmotic shock of *E. coli* cells harboring either pMET1 or pJHCMW1. The acetyltransferase activities from both strains behaved identically in an anion-exchange column. The only difference was that in the extract of *E. coli* carrying pJHCMW1, which has a higher copy number than pMET1, the level of activity was higher (data not shown).

Analysis of the pMET1 restriction endonuclease map indicated that coordinates 0 to 3.5 kbp (Fig. 2) were identical to the map of Tn*3* where the genes *tnpA* and *tnpR* are located. Besides, hybridization experiments using the Tn*3* *tnpR* region as a probe demonstrated that pMET1 carries three regions homologous to *tnpR* centered at coordinates 3.5, 6.5, and 9.5 kbp (Fig. 2). Mapping of the pMET1 DNA region carrying genetic determinants for resistance to ampicillin cloned in pPEM1 (Fig. 2) produced a restriction endonuclease map identical to that of the β-lactamase gene from Tn*3*.

Compatibility of pMET1 and pJHCMW1. To determine whether the replication systems of plasmids pJHCMW1 and pMET1 are related, we investigated whether pMET1 is compatible with pJHCMW1. To perform this experiment, we used a derivative of pJHCMW1, pMET14, and a derivative of pMET1, pROX2. Both plasmids were stably maintained in *E. coli*, indicating that they are compatible. To confirm that the replication regions of pMET1 and pJHCMW1 are unrelated, we performed a hybridization experiment. The replication fragment of pJHCMW1 was isolated from pROXT1 and used as a probe against *Bam*HI-digested pMET1 and pJHCMW1 DNAs. The result of this experiment is shown in Fig. 1c. No hybridization was found between the probe and pMET1 DNA. Instead, the control pJHCMW1 showed strong hybridization, as expected. Therefore, the results demonstrated that the two replicons are not related.

Identification of Tn*1331.2*. To determine whether there was a transposition element in pMET1, we first generated a Tra^- derivative of pMET1 which carries a *cat* gene, pPEM10 (Fig. 2). Plasmids pPEM10 (possessing the putative transposon) and pVK102 (as a target for the putative transposon) were used to transform *E. coli* HB101. The transformed strain, *E. coli* HB101(pPEM10, pVK102) was mated in a triparental conjugation with *E. coli* C2110 as the recipient and *E. coli* HB101(pRK2013) as a helper to provide the Tra functions to mobilize pVK102. Transconjugants were selected on tetracycline, amikacin, and nalidixic acid plates. The growing colonies were then tested for susceptibility to chloramphenicol to detect those colonies to which only a derivative of pVK102 harboring resistance to amikacin (and not pPEM10) was transferred. These colonies were also resistant to ampicillin, kanamycin, streptomycin, and tobramycin. Analysis of the plasmid contents of several of the transconjugants demonstrated that they harbored only one plasmid, identified as pVK102 with a DNA insertion of 11 kbp. This transposable extra DNA fragment corresponded to the pMET1 DNA region encompassing coordinates 0 to 11 kbp clockwise in the map shown in Fig. 2 and was designated Tn*1331.2*. *Bst*EII digestion of three representatives of pVK102::Tn*1331.2*, a pVK102::Tn*1331* derivative, and pVK102 is shown in Fig. 3a. Hybridization of the gel shown in Fig. 3a with radioactively labeled Tn*1331* sequences as a probe revealed that all internal *Bst*EII fragments from either Tn*1331* or Tn*1331.2* hybridized with the probe (Fig. 3b). Besides those fragments, there was also homology with a fragment of variable size in each derivative (Fig. 3b). This latter fragment, represented as fragment 1 in Fig. 3c, is the DNA fragment encompassing the *Bst*EII site located to the right of Tn*1331* or Tn*1331.2* and the *Bst*EII site of pVK102 (Fig. 3c). The variability of the size of fragment 1 in the different derivatives confirmed that Tn*1331.2* could transpose to different locations in pVK102. Digestion of the pVK102::Tn*1331.2* derivatives shown in Fig. 3 with *Sst*I produced the 3-kbp DNA fragment (data not shown) predicted from the map of the pVK102:Tn*1331.2* derivatives (Fig. 3c). As expected, this 3-kbp *Sst*I DNA fragment was absent in the derivative carrying a Tn*1331* copy.

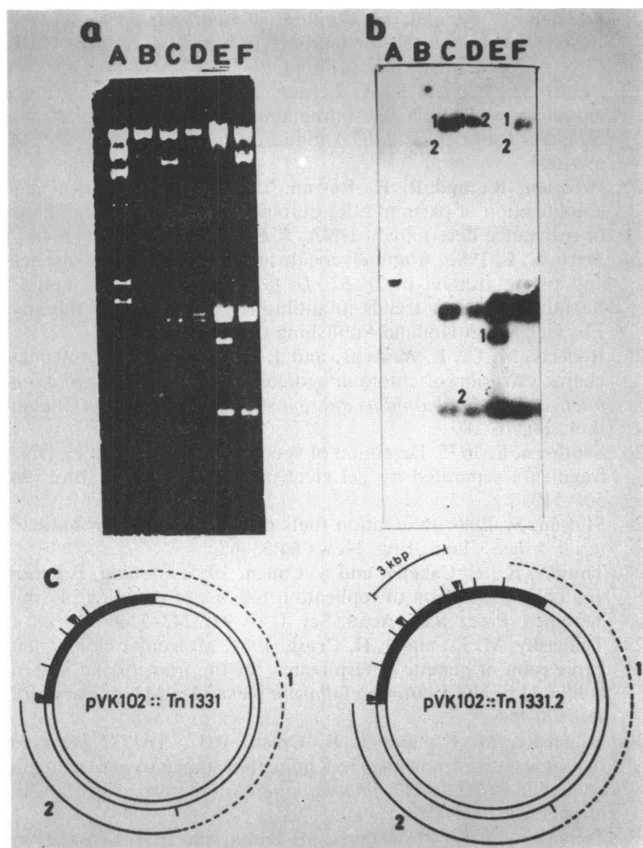

FIG. 3. Tn*1331* and Tn*1331.2* insertions in pVK102. (a) Agarose gel electrophoresis of *Bst*EII-digested representatives of the pVK102::Tn*1331* (lane C) and pVK102::Tn*1331.2* (lanes D to F) derivatives. Lane B contained *Bst*EII-digested pVK102, and lane A contained *Hin*dIII-digested lambda DNA. (b) Autoradiograph of Southern blot hybridization of the gel in panel a with ^{32}P-labeled Tn*1331* DNA sequences isolated from pJHCMW1 as a probe. The numbers 1 and 2 indicate the fragments represented in panel c. (c) *Bst*EII (|) and *Sst*I (▼) maps of pVK102::Tn*1331* and pVK102:: Tn*1331.2* derivatives. Plasmid pVK102 is represented by an open bar, and Tn*1331* and Tn*1331.2* are indicated by a filled bar. Fusion fragment 1 is indicated by a dotted line, and fragment 2 is shown by a solid line.

DISCUSSION

Amikacin is refractory to most inactivating enzymes, and a low incidence of resistance has been an important feature of this aminoglycoside (27). However, resistant strains have already been found (11, 12, 19, 22, 33, 34). From a pathogenic *K. pneumoniae* isolate, we recently isolated a transposition sequence, Tn*1331*, present in the 11-kbp plasmid pJHCMW1, carrying determinants for amikacin resistance (30). A serious outbreak of nosocomial infection occurred in May 1986 in Mendoza, Argentina. We isolated a 48-kbp plasmid, pMET1, from *K. pneumoniae* FC1 isolated from the diseased children. This plasmid was compared with pJHCMW1. Incompatibility experiments and comparison of the replication regions of both plasmids demonstrated that they are unrelated. However, molecular cloning and analysis of the 3-kbp *Bam*HI region carrying the genetic determinants for resistance to amikacin in pMET1 demonstrated that it is identical to that in pJHCMW1, and as expected from these results, both plasmids encode acetyltransferases which behaved identically when subjected to ion-exchange chromatography. It was of interest that such a *Bam*HI fragment was found duplicated in tandem in pMET1. Since the DNA regions surrounding this DNA fragment in pMET1 were identical to those present in the transposon carried by pJHCMW1, suggesting the presence of a transposition element belonging to the Tn*3* family, such as Tn*1331*, experiments to determine whether there was a transposition sequence in pMET1 were performed. A transposon, Tn*1331.2* (coordinates 0 to 11 kbp in Fig. 2), was identified by using a mating procedure. In a *recA* background, Tn*1331.2* was transposed from a pMET1 derivative to pVK102. A comparison of the physical maps of Tn*1331* and Tn*1331.2* demonstrated that the only difference is the presence of the duplication in tandem of the 3-kbp *Bam*HI fragment carrying the amikacin resistance gene in Tn*1331.2*. Another difference due to this duplication was the presence of three regions homologous to the Tn*3* *tnpR* gene in Tn*1331.2*; only two are present in Tn*1331*. Since in the transposition experiments all of the transconjugants harbored only pVK102:: Tn*1331.2* and not pPEM10, the resolution of the cointegrate must have taken place in the original *E. coli* HB101(pVK102, pPEM10), indicating that Tn*1331.2* carries at least one functional copy of *tnpR*. We do not know whether all three regions are able to code for a functional resolvase. Whether duplication of the fragment carrying the acetyltransferase gene confers an advantage to cells harboring Tn*1331.2* compared with cells carrying Tn*1331* in a similar copy number is presently being analyzed. It is possible that Tn*1331* can be converted to Tn*1331.2* and vice versa in a phenomenon similar to the transition and back-transition described for the neomycin-kanamycin resistance gene of plasmid NR1 (23) or those described for amplification of chloramphenicol and tetracycline resistance genes (17, 18). Experiments to find out whether Tn*1331* can duplicate its 3-kbp *Bam*HI fragment to become Tn*1331.2* are being performed.

Results presented in this work, i.e., showing that two unrelated plasmids from pathogenic *K. pneumoniae* strains from distant geographical regions carry related transposable elements, are consistent with epidemiological spread of amikacin resistance mediated by transposition.

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