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Tn*1331*, a Novel Multiresistance Transposon Encoding Resistance to Amikacin and Ampicillin in *Klebsiella pneumoniae*

MARCELO E. TOLMASKY[1,2] AND JORGE H. CROSA[1]*

Department of Microbiology and Immunology, School of Medicine, The Oregon Health Sciences University, Portland, Oregon 97201,[1] and Instituto de Investigaciones Bioquimicas "Fundacion Campomar," Facultad de Ciencias Exactas y Naturales, and CONICET, 1405 Buenos Aires, Argentina[2]

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A 7.5-kilobase-pair multiresistance transposon, Tn*1331*, harboring amikacin resistance was identified as part of *Klebsiella pneumoniae* plasmid pJHCMW1. Restriction mapping, hybridization, and transposition complementation experiments demonstrated that Tn*1331* belongs to the Tn*3* family. Its structure is similar to that of Tn*3* with the insertion of a DNA fragment encoding resistance to amikacin, kanamycin, and tobramycin.

Klebsiella pneumoniae is an important nosocomial pathogen. Antibiotic-resistant strains of *K. pneumoniae* are frequently found in the hospital environment (1). Resistance to aminoglycosides in particular has led to the introduction of semisynthetic derivatives, such as amikacin which is refractory to most inactivating enzymes (14). However, chromosome- and plasmid-mediated resistance to amikacin has already been detected in various gram-negative bacteria, including *K. pneumoniae* (6, 12, 13, 17–21, 26–28).

We recently examined a multiresistant *K. pneumoniae* strain in which resistance to amikacin (Ak^r), as well as resistance to ampicillin (Ap^r), resistance to kanamycin (Km^r), and resistance to tobramycin (Tm^r), was encoded by an 11-kilobase-pair (kbp) plasmid, pJHCMW1 (28). Molecular cloning and mutagenesis analysis of the pJHCMW1 Ak^r gene suggested that Km^r and Tm^r are encoded by this gene, while the Ap^r gene is localized in a different region of pJHCMW1 (26).

In this work, we describe the identification of a multiresistance transposon, Tn*1331*, of 7.5 kbp which harbors the Ak^r and Ap^r genes of *K. pneumoniae* plasmid pJHCMW1.

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MATERIALS AND METHODS

Bacterial strains and plasmids. *Escherichia coli* HB101 *pro leu thi lacY hsr hsm endA recA* (4), *E. coli* C2110 *polA gyrA* (7), and *K. pneumoniae* JHCK1 (28) have already been described. The plasmids used are listed in Table 1.

Chemicals. The following antibiotic concentrations were used for selection: ampicillin, 1 mg/ml; amikacin, 25 μg/ml; kanamycin, 25 μg/ml; tetracycline, 20 μg/ml; tobramycin, 25 μg/ml; gentamicin, 25 μg/ml; streptomycin, 50 μg/ml; and nalidixic acid, 50 μg/ml. Colicin E1 (Sigma Chemical Co., St. Louis, Mo.) susceptibility was determined as recommended by the supplier.

Isolation of plasmid DNA and restriction endonuclease analysis. Plasmid DNA was prepared as previously described (3, 28). Restriction endonuclease cleavage of plasmid DNA was done under the conditions recommended by the supplier (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). Electrophoresis of cleaved DNA was performed in a horizontal 0.7% (wt/vol) agarose gel (28).

Conjugation and transformation. Conjugations were performed at 37°C as previously described (28). Transformation with plasmid DNA was performed by the method of Cohen et al. (5).

Southern blot hybridizations and labeling of plasmid DNA. Plasmid DNA was extracted from agarose gels (2) and labeled by nick translation using [α-^{32}P]dCTP (22). Restriction fragments were transferred to nitrocellulose paper by the Southern blot transfer method (25). Hybridization experiments were performed as described previously (28). The filters were exposed to Kodak XAR-5 X-ray film.

RESULTS

Homology of pJHCMW1 regions with the Tn*3* transposition sequence. The 11-kbp multiresistance plasmid pJHCMW1 was isolated from *K. pneumoniae* (28) and transformed in *E. coli* HB101. It was stably maintained in this strain but was unable to replicate in *polA* mutants, such as *E. coli* C2110. A restriction map of pJHCMW1 was obtained by digestion of pJHCMW1 and various recombinant clones containing regions of this plasmid (Fig. 1). Plasmid pJHCMW1 had no

TABLE 1. Plasmids

Plasmid	Relevant genotype or phenotype[a]	Reference
pJHCMW1	Ak Ap Km Tm; 11-kbp *K. pneumoniae* plasmid	28
pVK102	Km Tc. RK2 replicon	16
pRK2013	Km. ColE1 replicon	8
pJHC-T15.2	Ak Ap Cm Km Tm; pJHCMW1 Ak gene cloned in pBR325	26
pJHC-T26.3	Ap Km; pJHCMW1 Ap gene cloned in a Km Ap derivative of pAT153	This work
RSF2124	Ap ColE1 with a Tn*3* insertion	24
RSF1050Δ511	Ap ColE1^{imm}, deletion derivative of RSF1050 in the *tnpA* gene resulting in Tn*3*Δ511	9
pMET14	Ak Km Tm Ap^s; deletion derivative of pJCHMW1	This work

[a] Ak, Amikacin resistance; Ap, ampicillin resistance; Cm, chloramphenicol resistance; Km, kanamycin resistance; Tc, tetracycline resistance; Tm, tobramycin resistance; Ap^s, ampicillin susceptibility; ColE1^{imm}, ColE1 immunity.

* Corresponding author.

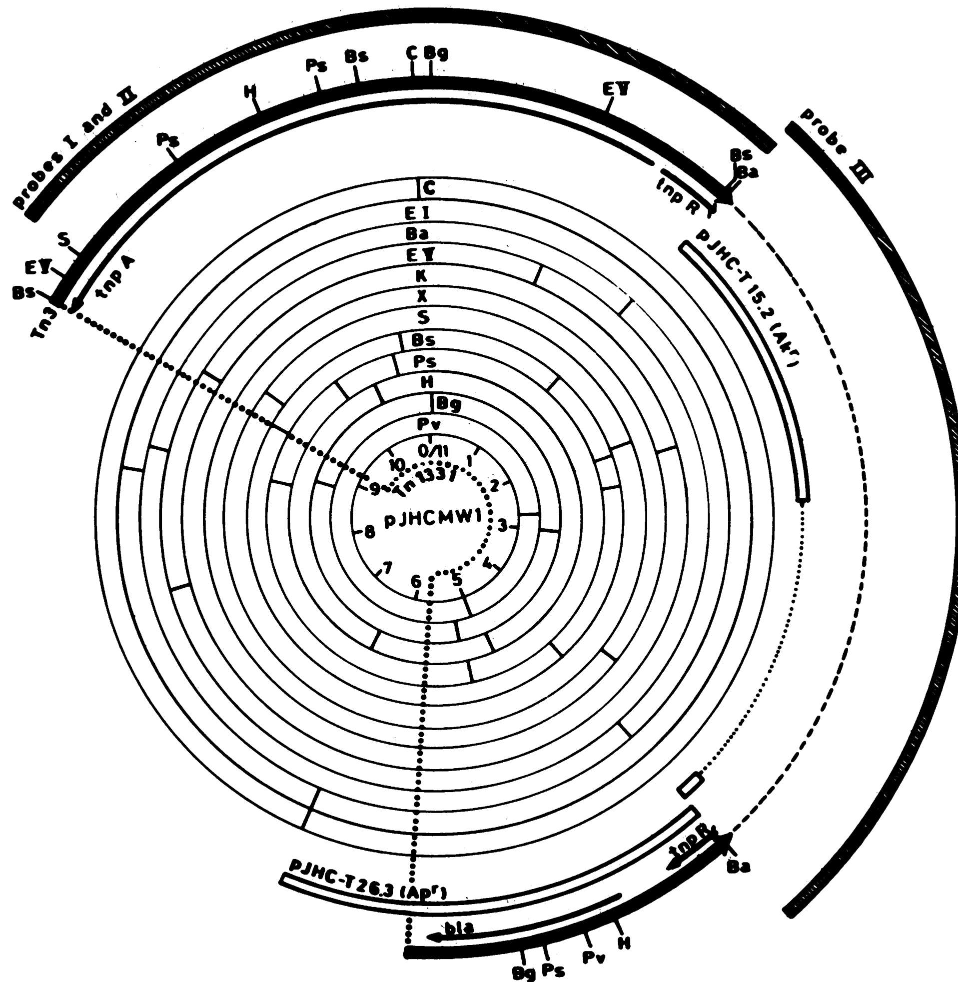

FIG. 1. Genetic and physical maps of pJHCMW1 and Tn*3*. The diagram shows the cleavage sites on plasmid pJHCMW1 for restriction enzymes *Cla*I (C), *Eco*RI (EI), *Bam*HI (Ba), *Eco*RV (EV), *Kpn*I (K), *Xho*I (X), *Sst*I (S), *Bst*EII (Bs), *Pst*I (Ps), *Hin*cII (H), *Bgl*I (Bg), and *Pvu*I (Pv). (The plasmid has no sites for *Hin*dIII, *Bgl*II, *Sal*I, *Sma*I, and *Xba*I.) Coordinates are given in kilobase pairs relative to one of the *Cla*I sites. The pJHCMW1 map is matched with the Tn*3* restriction map adapted from Heffron et al. (9–11). The pJHCMW1 restriction map was obtained by a combination of double digestions and molecular cloning of partial digests of pJHCMW1 DNA. The dotted lines crossing the pJHCMW1 map indicate the ends of Tn*1331*. The open bars show pJHCMW1 DNA fragments cloned in pJHC-T15.2 and pJHC-T26.3 with their relevant genotypes. The dotted line in pJHC-T15.2 represents a deleted DNA fragment from the original clone pJHC-T00181 that included the whole pJHCMW1 *Bam*HI fragment (26). The solid bar represents Tn*3* DNA. To facilitate the comparison with the restriction map of pJHCMW1, drawing of the Tn*3* DNA map was interrupted in the *Bam*HI site of the *tnpR* gene and continued at the location of pJHCMW1 coordinate 4.3 kbp. Both fragments of Tn*3* DNA are connected by a dashed line. The lines below the Tn*3* restriction map represent the genes *tnpA* (transposase), *tnpR* (resolvase), and *bla* (β-lactamase) (9–11). Hatched bars show DNA fragments used as probes. Probes I (from Tn*1331*) and II (from Tn*3*) were obtained by isolation of the DNA fragment after digestion of pJHCMW1 (probe I) or RSF2124 (probe II) with *Bam*HI and *Sst*I. Probe III was obtained after isolation of the 3-kbp DNA fragment generated by *Bam*HI digestion of pJHCMW1.

restriction sites for *Hin*dIII, *Bgl*II, *Sal*I, *Sma*I, and *Xba*I. Mapping of the pJHCMW1 Ap^r region cloned in pJHC-T26.3 produced a restriction map identical to that of β-lactamase from Tn*3* (Fig. 1). The regions were also homologous as determined by Southern blot hybridization between DNA from the β-lactamase gene of Tn*3* and the DNA region encompassing the Ap^r gene in pJHCMW1 (data not shown). A comparison of the restriction maps of the DNA regions of *tnpA* and *tnpR* genes in Tn*3* (10, 15) and the pJHCMW1 DNA fragment from coordinate 9.1 to 1.3 kbp (clockwise, Fig. 1) led to the conclusion that they are identical (Fig. 1). Probes encompassing the 3.3-kbp DNA fragment from the *Sst*I to *Bam*HI sites in pJHCMW1 (probe I, Fig. 1) and in Tn*3* (probe II, Fig. 1) were hybridized against restriction endonuclease-cleaved DNA from RSF2124, which is the ColE1 plasmid containing Tn*3* (24), and from pJHCMW1 (Fig. 2). The results of this experiment showed that both probes behaved identically. They showed homology against RSF2124 digested with *Bam*HI and with the 3.65-kbp *Bam*HI fragment of pJHCMW1 (Fig. 2b and c, lanes A and D). They also showed extensive homology against the 3.3-kbp fragment generated by digestion of either pJH CMW1 or RSF2124 with *Bam*HI plus *Sst*I (Fig. 2b and c, lanes B and E), indicating that this DNA fragment, which encompasses most of the *tnpA* gene and part of the *tnpR* gene in Tn*3*, is homologous in both plasmids. Digestion of pJHCMW1 or RSF2124 with *Bst*EII and hybridization of the fragments against probe I or II resulted in a radioactive band in the position where the two *Bst*EII fragments of 1.7 kbp present in Tn*3* and also in pJHCMW1 coband (Fig. 2b and c,

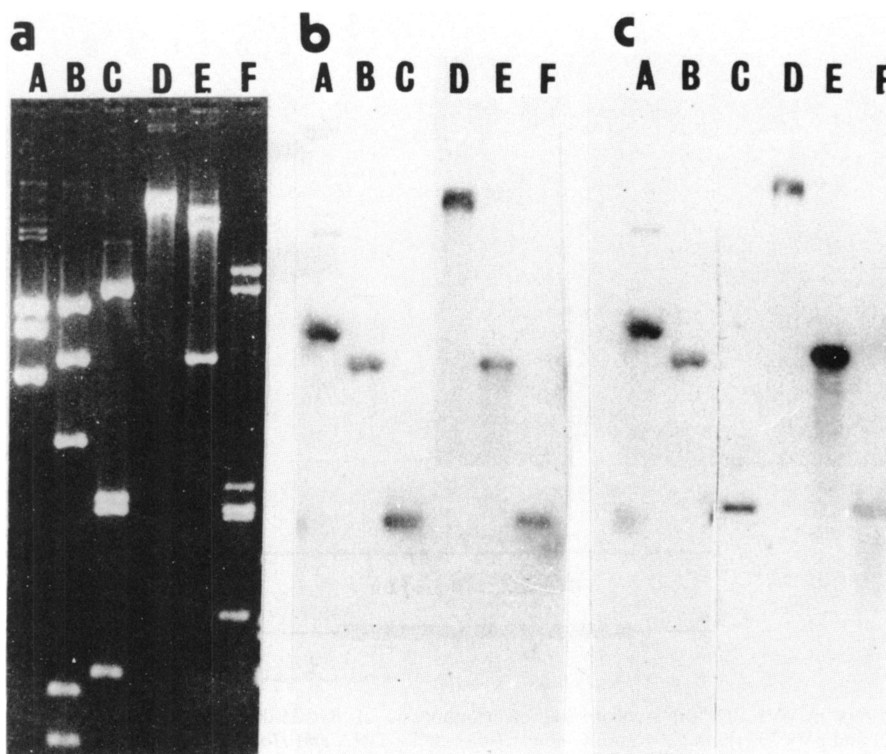

FIG. 2. Restriction endonuclease cleavage and hybridization of pJHCMW1 and RSF2124. (a) Agarose gel electrophoresis of plasmid DNA from pJHCMW1 (lanes A, B, and C) and RSF2124 (lanes D, E, and F) digested with *Bam*HI (lanes A and D), *Bam*HI plus *Sst*I (lanes B and E), and *Bst*EII (lanes C and F). (b) and (c) Autoradiographs of Southern blot hybridizations of gels identical to that in panel a using probes I (b) and II (c).

lanes C and F; also see map in Fig. 1). Conversely, hybridization of *Bam*HI-digested RSF2124 against the 3-kbp pJHCMW1 *Bam*HI fragment (probe III in Fig. 1) as a probe gave a low level of homology (data not shown). Probe III carries the genetic determinants encoding Ak^r in pJHCMW1 (Fig. 1) (26). The very weak homology between probe III and RSF2124 could have been due to a short stretch of DNA common to both plasmids beyond the *Bam*HI site in pJHCMW1. This is represented in Fig. 1 by an arrowhead. As expected, hybridization of the control *Bam*HI-digested pJHCMW1 with probe III gave a very strong radioactive signal in the location of the 3-kbp fragment (data not shown).

Ak^r gene in pJHCMW1 is part of a transposon. The homology observed between Tn*3* and regions of pJHCMW1 suggested the possibility of a transposition sequence in this plasmid. Identification of this transposition sequence was performed by a mating procedure using as a target DNA for the transposon plasmid pVK102, which can replicate in *polA* strains of *E. coli*. Since pJHCMW1 cannot replicate in *polA* mutants of *E. coli*, utilization of *E. coli* C2110 (*polA*) as recipient permitted us to select for pVK102 derivatives carrying the transposon. *E. coli* HB101 *recA*(pVK102) was transformed with pJHCMW1 DNA. Any transposition event from pJHCMW1 to pVK102 occurring in this strain was detected by mating it with a recipient *E. coli* C2110 (*polA*) using *E. coli* HB101 *recA*(pRK2013) as helper for mobilization of pVK102. Selection was performed in L agar containing nalidixic acid, tetracycline, and amikacin or nalidixic acid and tetracycline. Several hundred colonies growing in the presence of nalidixic acid, tetracycline, and amikacin were tested and found to also be resistant to kanamycin, tobramycin, and ampicillin. The ratio between the amount of colonies resistant to nalidixic acid, tetracycline, and amikacin and the amount of those resistant to nalidixic acid and tetracycline after the conjugation experiment was 7.7×10^{-5}. This number indicated the frequency of transposition of the pJHCMW1 DNA region carrying Ak^r and Ap^r into pVK102. Analysis of the cells resistant to nalidixic acid, ampicillin, amikacin, kanamycin, and tobramycin did not show any plasmid corresponding to pVK102. The transposition of Tn*1331* is *recA* independent, since transposition occurred in the *recA* strain before selection in the final *polA* recipient (10, 15). Restriction endonuclease and hybridization analysis of these derivatives demonstrated that these derivatives were pVK102 with a 7.5-kbp insertion. This extra DNA fragment corresponded to the pJHCMW1 DNA region encompassing coordinates 9.2 to 5.7 kbp clockwise in the map shown in Fig. 1. We designated this transposable DNA element Tn*1331*. The *Bst*EII digestion patterns of representatives of these derivatives (from now on called pVK102::Tn*1331*) and of pVK102 are shown in Fig. 3a. The latter plasmid has one site for *Bst*EII while Tn*1331* has six, one of them almost at its end (see map, Fig. 1). Figure 3a shows that digestion of pVK102::Tn*1331* DNA with *Bst*EII generates five constant DNA fragments internal to Tn*1331* DNA and two fragments of various sizes (fragments 1 and 2, Fig. 3c). One of these variable fragments encompassed the Tn*1331* *Bst*EII site located to the right in Fig. 3c and the *Bst*EII site in pVK102 (fragment 1, Fig. 3c). The other variable fragment comprehended the *Bst*EII site in pVK102 and the *Bst*EII site located almost to the end of Tn*1331* (fragment 2, Fig. 3c). The smallest of the Tn*1331* *Bst*EII

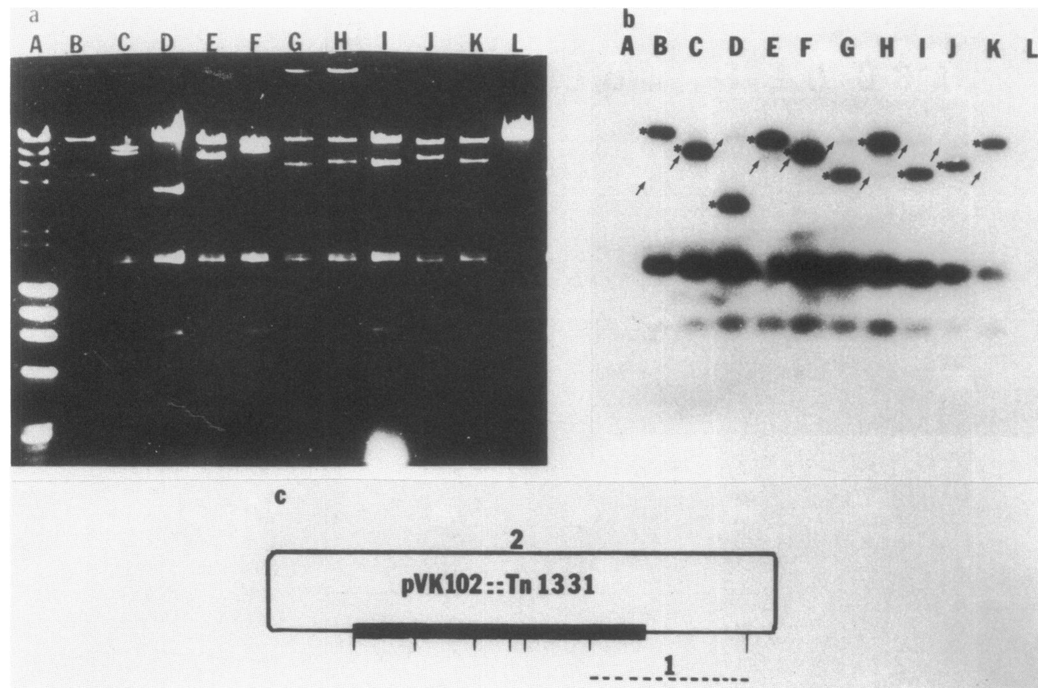

FIG. 3. Tn*1331* insertions in pVK102. (a) Agarose gel electrophoresis of *Bst*EII-digested representatives of the pVK102::Tn*1331* derivatives (lanes B to K) and pVK102 (lane L). Lane A, *Hin*dIII-digested λ DNA and *Hae*III-digested φX174 DNA. (b) Autoradiograph of Southern blot hybridization of the gel in panel a using ^{32}P-labeled pJHCMW1 DNA as a probe. The asterisks and arrows show the positions of fusion fragments 1 and 2, respectively. Fusion fragment 1, indicated by a dashed line in panel c, is that formed between the *Bst*EII site on the right side in Tn*1331* and the *Bst*EII site in pVK102. Fusion fragment 2 encompasses the DNA fragment between the *Bst*EII site in pVK102 and the *Bst*EII site located almost at the left end of Tn*1331* DNA. In lanes C and F, fragments 1 and 2 band very close to each other and consequently fragment 2 cannot be seen due to the level of the radioactive signal of fragment 1. (c) Diagram of a *Bst*EII restriction map of a pVK102::Tn*1331* derivative; pVK102 DNA is represented by a thin line, and Tn*1331* DNA is represented by a thick line. Fusion fragments 1 and 2 are indicated.

fragments (0.4 kbp) was not detected in the gel shown in Fig. 3. Southern blot hybridization of the gel shown in Fig. 3a using pJCHMW1 DNA as a probe showed strong homology with *Bst*EII pVK102::Tn*1331* fragment 1 (Fig. 3b, lanes B to K, bands with asterisks). As expected, fragment 2 showed little homology with the probe (Fig. 3b, lanes B to K, bands with arrows). The various sizes of fragments 1 and 2 in the pVK102::Tn*1331* derivatives demonstrated the ability of Tn*1331* to insert at different locations in pVK102.

Complementation of *tnpA* mutant of Tn*3* with a product made by Ak^r transposon Tn*1331*. To further prove that Tn*1331* is indeed related to Tn*3*, we determined whether a tranposase-deficient mutant of Tn*3*, Tn*3*Δ511 present in plasmid RSF1050Δ511 (9), could be complemented with a product of Tn*1331* which, as shown in Fig. 1, contains a sequence that corresponds to the Tn*3* *tnpA* gene. For this task, we generated a Tn*1331* derivative with an intact *tnpA* region but with a lesion in its ability to transpose. Deletion of the smallest *Hin*cII DNA fragment from pJMCMW1 (see map in Fig. 1) generated plasmid pMET14 which lacks most of the β-lactamase gene, as well as one of the ends of Tn*1331*. To assess whether the deleted Tn*1331* could not transpose, *E. coli* HB101(pVK102) was transformed with pMET14 to determine the frequency of transposition of Ak^r from this plasmid to pVK102. The results showed that the frequency of transposition of Ak^r dropped drastically compared with that from pJHCMW1 (from 3×10^{-4} to less than 10^{-9}). Hence, pMET14 was used as a source of Tn*1331* *tnpA*-like product in a complementation experiment with RSF1050Δ511 (11), a derivative of RSF1050 with a deletion affecting the Tn*3* *tnpA* gene. The experiment was done by mating an *E. coli* HB101 *recA* strain carrying pMET14, RSF1050Δ511, and pVK102 (as a target) with *E. coli* C2110 *polA* as recipient strain and *E. coli* HB101 *recA*(pRK2013) to provide a helper for mobilization of pVK102. The results indicated that the presence of pMET14 increased dramatically the frequency of transposition of Ap^r from RSF1050 Δ511 to pVK102 (from 5×10^{-9} to 6×10^{-5}), demonstrating that the Tn*3*Δ511 mutant could transpose efficiently in the absence of the *E. coli recA* gene using the *tnpA*-like product from Tn*1331*. All *E. coli* transconjugants were susceptible to colicin E1, indicating that in all pVK102::Tn*3*Δ511 derivatives present in these transconjugants, resolution of the cointegrate took place (data not shown).

DISCUSSION

Genes specifying resistance to aminoglycosides may be included in transposable elements (15); consequently, experiments were performed to determine whether *K. pneumoniae* plasmid pJHCMW1 harbored a transposon encoding Ak^r. We showed in this work that the pJHCMW1 DNA region encompassing coordinates 9.2 to 1.3 and 4.3 to 5.7 kbp had restriction cleavage sites identical to those of Tn*3* and that the *tnpA*, *tnpR*, and *bla* genes of Tn*3* have homology with those pJHCMW1 regions. The DNA fragment encompassing coordinates 1.3 to 4.3 kbp carries the gene encoding resistance to amikacin. A transposable sequence, designated Tn*1331*, carrying Ap^r and Ak^r (this gene also encodes resistance to Tm^r and Km^r) was identified in

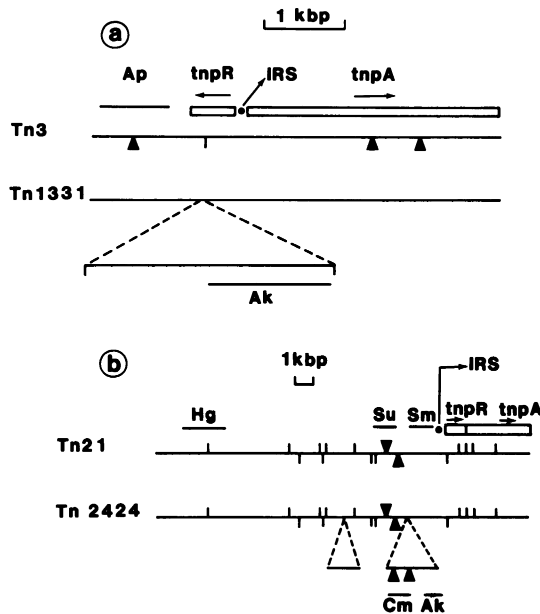

FIG. 4. Comparison of physical and genetic maps of transposons Tn*21*, Tn*2424*, Tn*3*, and Tn*1331*. (a) Restriction endonuclease maps of Tn*3* and Tn*1331* showing the extra fragment present in Tn*1331*. (b) Comparative restriction endonuclease maps of Tn*21* and Tn*2424* showing the two extra DNA pieces present in Tn*2424* with respect to Tn*21* as described by Meyer et al. (18). The arrows on top of the boxes indicating *tnpA* and *tnpR* genes show transcriptional orientation. IRS, Internal resolution site (10, 11); the size of the dot was not drawn to scale. Resistances to antimicrobial agents and restriction sites are also indicated in the maps: Cm, chloramphenicol resistance; Ak, amikacin resistance; Su, sulfonamide resistance; Sm, streptomycin resistance; Hg, mercuric salts resistance; Ap, ampicillin resistance. *Bam*HI is indicated by vertical lines pointing down; *Eco*RI is indicated by vertical lines pointing up. ▼, *Sal*I; ▲, *Pst*I. No sites for *Sal*I and *Eco*RI are present in Tn*3*.

pJHCMW1 by using a mating procedure. Tn*1331* was transposed from pJHCMW1 to plasmid pVK102 in *recA* strain HB101, therefore demonstrating *recA* independence of this transposition event. Mapping of the transposed DNA fragment demonstrated that Tn*1331* encompassed coordinates 9.2 to 5.7 kbp (clockwise in Fig. 1) in pJHCMW1. A comparison of the physical maps of Tn*3* and Tn*1331* suggests that in Tn*1331* the DNA fragment carrying genetic determinants for Ak^r (and also Km^r and Tm^r) is inserted between the *tnpA* and *bla* genes of Tn*3*. In the tranposition experiments, done in the *recA* strain, HB101, no cointegrate was isolated; therefore, Tn*1331* must have a functional *tnpR* gene and transposition occurs independent of the *recA* product. Inspection of the physical map of pJHCMW1 shows that approximately at coordinates 1.3 and 4.3 kbp there are *Bam*HI and *Bst*EII sites at the position found within the *tnpR* gene in Tn*3* (Fig. 1). For the sake of simplicity, in Fig. 1 the *tnpR* gene of Tn*3* was drawn as if one part ended at coordinate 1.3 kbp and the other part started at coordinate 4.3 kbp, although we are uncertain as to the location of the complete copy of *tnpR* on pJHCMW1. Furthermore, we cannot rule out the presence of two complete copies, each flanking the 3-kbp fragment containing the Ak^r determinant. Recent evidence from our laboratory indicates that sequences upstream of the Ak^r gene are indeed homologous to the end of the *tnpR* gene and that the Ak^r gene is under the control of sequences identical to the transcriptional and translational start signals of the β-lactamase gene, with the first six amino acids of the acetyltransferase gene responsible for Ak^r being identical to those of the Tn*3* β-lactamase gene (K. Nobuta, M. Tolmasky, L. Crosa, and J. H. Crosa, submitted for publication). Thus, Tn*3*-like sequences may play a role not only in the ability of the amikacin resistance gene to be spread epidemiologically but also in the control of its expression.

Two main groups of Tn*3*-related transposable elements have been described: the Tn*21* subgroup and the Tn*3*-proper subgroup (18, 23). Transposable elements belonging to the Tn*21* subgroup are characterized by having both genes *tnpA* and *tnpR* in the same transcriptional orientation, with the internal resolution site located upstream of the *tnpR* gene (Fig. 4). The other subgroup, with examples such as Tn*3* and Tn*1000*, has the internal resolution site between the *tnpR* and *tnpA* genes which are transcribed in opposite orientations (Fig. 4) (10, 11, 18). Our restriction endonuclease mapping and hybridizations suggest that Tn*1331* not only is related to Tn*3* but also that as it occurs in Tn*3*, the *tnpR* and *tnpA* genes show opposite transcriptional orientations (Fig. 4). Consequently, this multiresistance transposon must belong to the Tn*3* subgroup and may have arisen as an evolutionary step. The acquisition of resistance genes by Tn*21*-like transposable elements has been described previously (18, 23). A Tn*21*-like multiresistance transposon, Tn*2424*, carrying determinants for Ak^r was recently identified in *E. coli* (18). Transposons Tn*1331* and Tn*2424* are not directly related, since the latter, in addition to Ak^r, carries determinants for resistance to mercuric salts, sulfonamide, and chloramphenicol but does not confer resistance to ampicillin. Therefore, although many Tn*21*-like transposons with extra pieces of DNA encoding resistance genes have been described, Tn*1331* from *K. pneumoniae* is the first example of resistance gene addition to a Tn*3*-like element.

Tn*1331* carries Ak^r in the same transposon along with Ap^r. Amikacin is normally used sparingly to reduce the selection of resistant strains, but the widespread use of ampicillin could also cause the dissemination of Ak^r determinants through Tn*1331* transposition.

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