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Amikacin Resistance Mediated by Multiresistance Transposon Tn*2424*

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Tn*2424*, a multiresistance transposon 25 kilobases long, was isolated from IncFII plasmid NR79. Tn*2424* transposed resistance to sulfonamides, streptomycin and spectinomycin, mercuric chloride, chloramphenicol, and amikacin with a frequency of 6×10^{-5}. Resistance to amikacin was mediated by a 6′-*N*-acetyltransferase, which conferred higher levels of resistance in *Pseudomonas aeruginosa* than in *Escherichia coli*. A restriction analysis and cloning experiments resulted in a physical and functional map of Tn*2424*. Comparison by a heteroduplex technique revealed that Tn*2424* includes the total sequence of Tn*21* and two additional DNA fragments that are 1.8 and 4 kilobases long.

Aminoglycoside resistance is known to be mediated by modifying enzymes (for reviews, see references 11 and 23). The semisynthetic aminoglycoside antibiotics amikacin and netilmicin have been developed because of their resistance to attack by these inactivating enzymes (25). Therefore, these antibiotics are highly potent drugs for the treatment of serious infections, particularly when the infecting organisms are resistant to other aminoglycosides. In gram-negative bacteria only one group of enzymes, the 6′-*N*-acetyltransferases, seems to confer resistance to these semisynthetic aminoglycoside derivatives (25). Despite the fact that several aminoglycoside-inactivating enzymes are encoded by transposable elements (18), until now no transposon that mediates resistance to amikacin was known.

In this paper we describe the isolation of a multiresistance transposon about 25 kilobases (kb) long, designated Tn*2424*, from IncFII plasmid NR79 (3). Besides resistance to mercuric chloride, sulfonamides, streptomycin and spectinomycin [AAD-(3″)], and chloramphenicol, this transposon codes for resistance to amikacin, netilmicin, and several other aminoglycoside antibiotics due to a 6′-*N*-acetyltransferase. A determination of the structure of Tn*2424* by restriction analysis and a hetero-duplex technique revealed its close relationship to Tn*21*, another multiresistance transposon that has been assumed to be involved in an evolutionary sequence of several transposable elements (13, 33).

MATERIALS AND METHODS

The bacterial strains and plasmids which we used are listed in Table 1.

Media and antibiotics. *Escherichia coli* was grown on Iso-Sensitest agar (Oxoid Ltd., Wesel, Germany) or on M9 minimal medium containing 2% (wt/vol) lactose. *Proteus mirabilis* transconjugants were selected on Simmons citrate agar (Oxoid). *Pseudomonas aeruginosa* was grown on Cetrimid agar (E. Merck AG, Darmstadt, Germany). The following antibiotic concentrations were used for selection: ampicillin, 50 mg/liter; amikacin, 8 mg/liter (for the selection of resistant *E. coli* and *Proteus mirabilis* strains) and 20 mg/liter (for the selection of resistant *Pseudomonas aeruginosa* strains); chloramphenicol, 30 mg/liter; paromomycin, 200 mg/liter; nalidixic acid, 100 mg/liter; sulfonamide, 250 mg/liter; spectinomycin, 50 mg/liter; streptomycin, 500 mg/liter; tetracycline, 20 mg/liter; mercuric chloride, 20 mg/liter.

Conjugal transfer of plasmids. Plasmid transfers among *E. coli*, *Proteus mirabilis*, and *Pseudomonas aeruginosa* were carried out by conjugation on filter disks.

Preparation of DNA and transformation procedure. DNA was prepared as described previously (8, 16, 29). The transformation procedure which we used has been described by Kushner (19).

Screening and digestion of cloned DNA fragments were performed by the following method. A 10-ml portion of an overnight culture containing the required antibiotics was centrifuged for 10 min, and the cells were suspended in 1 ml of 25% (wt/vol) sucrose–50 mM EDTA–50 mM Tris-hydrochloride (pH 8.0). Lysozyme (2 mg in water) was added, and the preparation was incubated for 15 min at 37°C. The cells were lysed by adding 1 ml of 10% (vol/vol) Triton X-100 in 50 mM EDTA–50 mM Tris-hydrochloride (pH 8.0) and were kept for 1 min in a boiling water bath. The centrifuge tubes were rapidly cooled on ice. The chromosomal DNA and proteins were removed by centrifugation at 15,000 × *g* for 10 min. The supernatant was mixed with 1 volume of isopropanol and then centrifuged for 10 min at 15,000 × *g*. The resulting pellet was suspended in 100 μl of TE buffer (10 mM Tris-hydrochloride, 1 mM EDTA, pH 8.0), and 25 to 50 μl of the suspension

TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant markers[a]	Reference
E. coli		
W677	*F⁻, thr, leu, thi, lac, mal, gal*	3
W3110	Nal^r, *lac*$^+$	14
JC2926	Sm^r, *recA, thi, thr, arg, his, leu, lac*	2
SK1592	*gal, thi, ton, sbcB15, hsr-4, hsm*$^+$	19
7118	*Δ(lac-pro), F'lacI^{q}ZΔM15 pro*$^+$	20
Proteus mirabilis Pm13		9
Pseudomonas aeruginosa PAO303	Rif^r, Nal^r, *arg-18*	24
Plasmids		
NR79	Tc^r, Su^r, Hg^r, Sm^r, Sp^r, Cm^r, Ami^r, Tra$^+$	3
pUB307	Pm^r, Tc^r, Tra$^+$	5
pBR322	Ap^r, Tc^r	6
pUR250	Ap^r	29
pUB307::Tn*2424*	Pm^r, Tc^r, Su^r, Hg^r, Sm^r, Sp^r, Ami^r, Cm^r, Tra$^+$	This paper
pBR322::Tn*2424*	Ap^r, Su^r, Hg^r, Sm^r, Ami^r, Cm^r	This paper
pBR322::Tn*21*	Ap^r, Su^r, Hg^r, Sm^r, Tc^r, Sp^r	Kratz[b]

[a] Nal^r, Nalidixic acid resistance; Sm^r, streptomycin resistance; Rif^r, rifampin resistance; Tc^r, tetracycline resistance; Su^r, sulfonamide resistance; Hg^r, mercuric chloride resistance; Sp^r, spectinomycin resistance; Cm^r, chloramphenicol resistance; Ami^r, amikacin resistance; Pm^r, paromomycin resistance; Ap^r, ampicillin resistance.

[b] J. Kratz, manuscript in preparation.

was used for screening plasmids. The residual DNA was dialyzed for 3 h at room temperature in TE buffer and then was extracted once with phenol and twice with ether. The DNA was precipitated with 1 volume of isopropanol and was centrifuged at 15,000 × *g* for 10 min. The resulting pellet was dried and dissolved in TE buffer, and 25 to 50 μl of the suspension was used for digestions.

Digestion and cloning of DNA. Endonuclease digestions and ligation reactions were carried out according to the instructions of the manufacturer (all enzymes were purchased from Boehringer, Mannheim, Germany). The method of selection for cloned DNA fragments by using isopropyl-β-D-thiogalactoside and 5-bromo-4-chloro-indolyl-β-D-galactoside (Sigma Chemical Co., Taufkirchen, Germany) has been described by Rüther (28).

Electron microscopy. The preparation of copper grids, the cytochrome *c* spreading technique, and heteroduplex preparation (hyperphase of 50% [vol/vol] formamide, 0.05 M Tris-hydrochloride [pH 8.0], and 0.05 M NaCl; hypophase of double-distilled water) were performed as described previously (12, 32, 34). The contour lengths of DNA molecules were measured with a Numonics digitizer (Nucleotron, Munich, Germany).

RESULTS

Isolation of Tn*2424*. *E. coli* W677 containing plasmids NR79 and pUB307 was mated with *Proteus mirabilis* Pm13, and transconjugants were selected for paromomycin and amikacin resistance on Simmons citrate agar. As *Proteus* transconjugants that had received pUB307::Tn*2424* yielded the same phenotype as transconjugants harboring pUB307 in addition to NR79, several transconjugants were screened for their plasmids by the method of Kado and Liu (16). No plasmid band corresponding to NR79 was detected, whereas four transconjugants contained one plasmid larger than pUB307. *E. coli* W3110 was mated with these *Proteus* clones (pUB307::Tn*2424*), with selection on M9-lactose minimal medium containing nalidixic acid, paromomycin, and amikacin. The resulting transconjugants [W3110(pUB307::Tn*2424*)] were tested for their resistance markers (Pm^r, Tc^r, Sp^r, Su^r, Hg^r, Ami^r, and Cm^r). One clone was mated with *E. coli* JC2926 (pBR322), and transconjugants were selected for streptomycin, paromomycin, and amikacin resistance. To elucidate the *rec* independence of the transposition event, the DNA of one resulting clone [*E. coli* JC2926(pUB307::Tn*2424* and pBR322)] was prepared (29) and used to transform *E. coli* SK1592. Transformants were selected for ampicillin and amikacin resistance. Several colonies were toothpick replicated and tested for the loss of paromomycin and tetracycline resistance. Tetracycline sensitivity indicated the loss of pUB307 and the insertion of the transposon into the *tet* region of pBR322. One tetracycline- and paromomycin-sensitive transformant clone of *E. coli* SK1592(pBR322::Tn*2424*) was used for the experiments described below.

Transfer of Tn*2424* from *E. coli* to *Pseudomonas aeruginosa*. To determine the transposition frequency of Tn*2424*, we transferred pBR322::Tn*2424* from *E. coli* SK1592 to *E. coli* JC2926 (pUB307). *E. coli* JC2926 (pBR322::Tn*2424* and pUB307) was mated with *Pseudomonas aeruginosa* PA0303. One-half of the *Pseudomonas* transconjugants were plated onto Cetrimid agar

TABLE 2. Levels of resistance mediated by pUB307::Tn*2424* in *E. coli* and *Pseudomonas aeruginosa*

Drug	Resistance level			
	E. coli W3110		*Pseudomonas aeruginosa* PAO303	
	No plasmid	pUB307::Tn*2424*	No plasmid	pUB307::Tn*2424*
Gentamicin	0.5[a]	1	0.5	1
Tobramycin	0.5	16	0.5	128
Sisomycin	0.5	4	0.5	32
Amikacin	0.5	16	0.5	64

[a] Highest antibiotic concentration (in milligrams per liter of Iso-Sensitest broth).

containing only paromomycin, and the second half were grown on Cetrimid plates that contained amikacin in addition to paromomycin. The resulting ratio of amikacin- and paromomycin-resistant colonies to paromomycin-resistant transconjugants was 6×10^{-5}; this value was considered for the transposition frequency. The resulting *Pseudomonas* transconjugants mediated higher levels of resistance than the *E. coli* strain to all of the substrates of 6′-*N*-acetyltransferase (Table 2). The resistance pattern shown in Table 2 (amikacin resistance and gentamicin sensitivity) clearly indicates that the aminoglycoside-inactivating enzyme belongs to group IV of the 6′-*N*-acetyltransferases according to the nomenclature of Mitsuhashi (22), as only this group is able to mediate resistance to amikacin in gram-negative bacteria.

Restriction analysis and physical map of Tn*2424*. The plasmid DNA of strain SK1592(pBR322::Tn*2424*) was used for single and double digestions with the following restriction enzymes: *Eco*RI, *Bam*HI, *Sal*I, *Sma*I, *Pst*I, and *Bgl*II. The restriction data resulted in a physical map of Tn*2424* (Fig. 1). *Eco*RI-*Bam*HI digestion revealed that Tn*2424* transposed into the *tet* gene adjacent to the *Bam*HI restriction site at about 0.25 kb in pBR322. Adding the sizes of all of the fragments resulted in a total length of nearly 25 kb for Tn*2424*.

Analysis of Tn*2424* by electron microscopy. The physical map of Tn*2424* led us to the conclusion that this transposable element is closely related to Tn*21*. Therefore, we determined the relationship between these two transposons by performing a heteroduplex analysis, using pBR322::Tn*21* and pBR322::Tn*2424* (Fig. 2A). As Tn*21* and Tn*2424* were inserted in opposite orientations in pBR322, hybridization of the two transposons left the vector portions single stranded in the heteroduplex molecule. The total sequence of Tn*21* is included in Tn*2424*, and, in addition, Tn*2424* contains two DNA fragments that are 1.8 and 4.0 kb long (Fig. 2B and C, loops 1 and 2). The two inserted fragments are separated by a 3.4-kb double-stranded region and do not show extended inverted repeats at their ends. Furthermore, the restriction enzyme patterns suggest that these elements are not flanked by directly repeated IS sequences.

Figure 2C shows that the additional DNA fragments of Tn*2424* are inserted into *Eco*RI fragments K and G of Tn*21*. The DNA between the coordinates at 9.8 and 11.6 kb (Fig. 1) represents loop 1 and carries an additional *Sma*I restriction site. The restriction sites for *Sma*I (coordinate at 15 kb) and *Eco*RI (coordinate at 19 kb) flank nearly all of the sequence representing loop 2.

Cloning experiments and functional map of Tn*2424*. To correlate the *aac* and *cat* resistance genes of Tn*2424* with the additional DNA segments (loops 1 and 2), we constructed a functional map of Tn*2424* (Fig. 1). *Eco*RI- and *Bam*HI-digested DNA of pBR322::Tn*2424* was cloned into pUR250 and used to transform *E. coli* 7118. White, ampicillin-resistant colonies

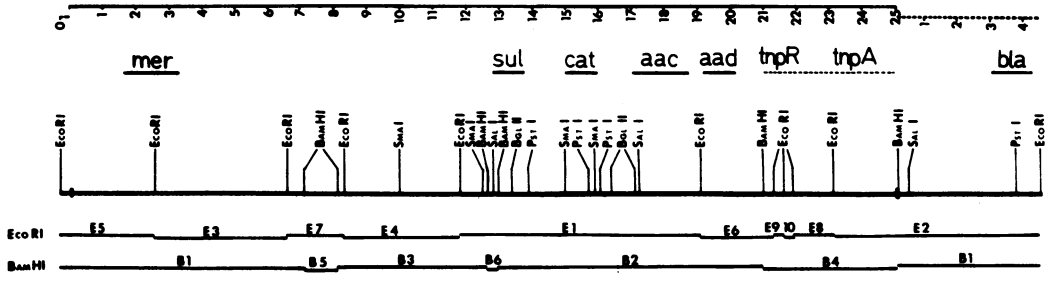

FIG. 1. Physical and functional map of pBR322::Tn*2424*. The scale at the top represents the length of plasmid pBR322 (broken lines) and the length of Tn*2424* (in kilobases). Tn*2424* is inserted adjacent to the *Bam*HI restriction site in the *tet* region of pBR322 at about 0.25 kb. The closed boxes flanking the transposable element indicate the inverted repeated ends (38 base pairs) of Tn*2424*. The gene designations correspond to the resistance markers for mercuric chloride (*mer*), sulfonamide (*sul*), chloramphenicol (*cat*), amikacin (*aac*), streptomycin and spectinomycin (*aad*), and ampicillin (*bla*) and to the genes for resolvase (*tnpR*) and transposase (*tnpA*).

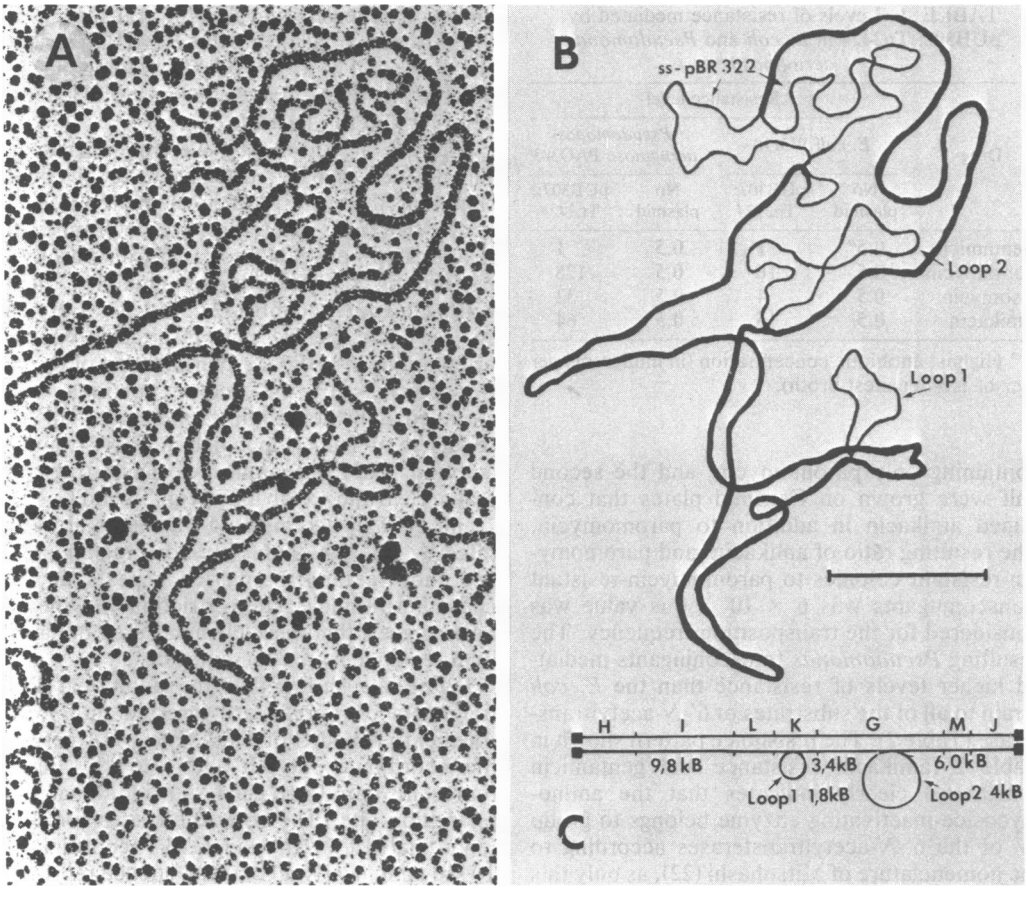

FIG. 2. (A) Heteroduplex formed by pBR322::Tn*21* and pBR322::Tn*2424*. (B) Schematic drawing of the heteroduplex molecule. Bar = 0.5 μm. (C) Schematic structures of Tn*21* (upper line) and Tn*2424* (lower line), with their fragments generated by *Eco*RI digestion. The solid boxes at the ends of Tn*21* and Tn*2424* correspond to the inverted repeated ends of both elements. The nomenclature of the *Eco*RI fragments of TN*21* has been described previously (7, 21).

showing at least one additional resistance marker of Tn*2424* were selected on Iso-Sensitest agar containing isopropyl-β-D-thiogalactoside and 5-bromo-4-chloro-indolyl-β-D-galactoside. Cloning experiments with *Eco*RI-digested DNA resulted in several Ap^r Su^r Cm^r colonies, whereas other cloned resistance markers, such as amikacin, spectinomycin, and mercuric chloride, were not detected. Screening of 15 Ap^r Su^r Cm^r colonies showed that *Eco*RI fragment E1 was present in all plasmids and therefore responsible for resistance to chloramphenicol and sulfonamides. Figure 3 shows that either self-ligated fragments of pBR332::Tn*2424* (in this case fragments E1 and E2) or *Eco*RI fragments E1 and the vector plasmid could be detected. The ampicillin resistance of clones containing ligated *Eco*RI fragments E1 and E2 was due to the presence of a part of pBR322 in fragment E2, as shown in Fig. 1. Cloning of *Bam*HI-digested DNA resulted in Ap^r Hg^r and Ap^r Ami^r Sp^r phenotypes. Besides the Ap^r Hg^r phenotype that was mediated by self-ligated *Bam*HI fragment B1, we determined the fragment that was responsible for spectinomycin and amikacin resistance. Screening of several Ap^r Ami^r Sp^r clones revealed that *Bam*HI fragment B2 carries both resistance genes (data not shown).

DISCUSSION

Several transposable elements conferring aminoglycoside resistance have been isolated (for a review of these elements, see reference 18). Despite the fact that resistance to modern, potent aminoglycoside antibiotics, such as gentamicin, tobramycin, and sisomicin, which are used in the treatment of serious infections, is known to be transposable (10, 26, 27), no transposon specifying amikacin resistance has been described.

We isolated Tn*2424* from IncFII plasmid NR79, which affects the bactericidal activities of a great number of aminoglycosides, including kanamycin, tobramycin, and sisomicin, as well as the semisynthetic derivatives amikacin and netilmicin. This activity is due to the production of 6'-*N*-acetyltransferase (3). In *Pseudomonas aeruginosa* this enzyme mediates to all of its substrates levels of resistance that are several times higher than those in *E. coli*. This observation will be discussed elsewhere (Meyer and Wiedemann, manuscript in preparation).

A comparison of the physical and functional maps, as well as electron microscopy, revealed that Tn*2424* is closely related to Tn*21* (which was derived from R100.1) (13), as Tn*2424* includes the total sequence of Tn*21*. This finding supports the results of Haas and Davies (15), who showed the striking relationship between plasmids NR79 and R100.

The *aac* gene of Tn*2424* is inserted together with the *cat* gene into *Eco*RI fragment G of Tn*21*, between the *sul* and *aad* genes (Fig. 2C). In this way the DNA segment carrying these additional resistance genes enlarges *Eco*RI fragment G by about 4 kb and leads to several additional restriction sites for *Sal*I, *Pst*I, *Bgl*II, *Eco*RI, and *Sma*I.

The failure to clone amikacin or spectinomycin resistance after *Eco*RI digestion led us to the assumption that *aac* and *aad* are related, perhaps by transcription from the same promoter. This transcription unit could be divided by the *Eco*RI site that separates fragments E1 and E6 (Fig. 1). A second inserted DNA fragment (length, 1.8 kb) is located in *Eco*RI fragment K of Tn*21*, resulting in an additional *Sma*I restriction site. This fragment lacks detectable functions. The locations of other functional genes of Tn*2424* were deduced from the homology between Tn*2424* and Tn*21* (Fig. 2). The mercury resistance gene has been located on *Eco*RI fragments E3 and E5 of Tn*2424* (*Eco*RI fragments H and I of plasmid NR1) according to the data of Miki et al. (21). The genes necessary for transposition (transposase and resolvase) have been located according to the positions of the corresponding genes of Tn*2603* (31) and Tn*21* (13). As Tn*2603* has been shown to be closely related to Tn*21* (33), we concluded that the transposase and resolvase are located within *Bam*HI fragment B4 of Tn*2424*, corresponding to fragment B2 of Tn*2603* (31). More precisely, *tnpA* is at the left side of the inverted repeat on a 3.1-kb DNA fragment (13, 31), which is capable of encoding a protein with a molecular weight of 110,000 (31). This region corresponds to *Eco*RI fragment E8 and a part of fragment E2 in Tn*2424*. Resolvase (*tnpR*) seems to be encoded by the DNA fragment adjacent to the left side of the *tnpA* region

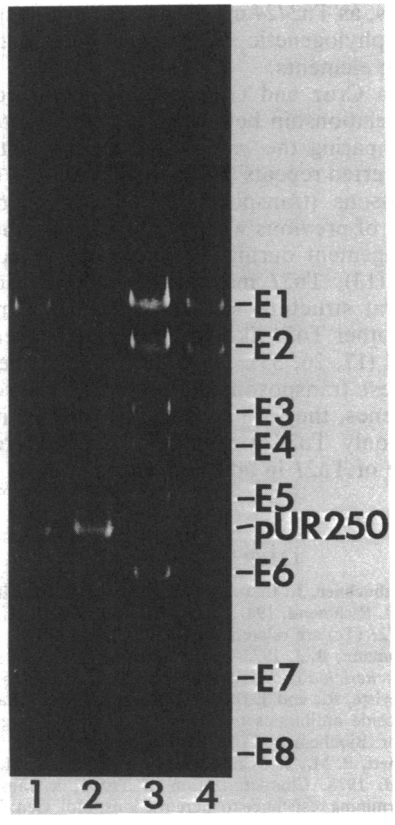

FIG. 3. Restriction analysis of plasmids from Ap^r Su^r Cm^r clones (lanes 1 and 4), pBR322::Tn*2424* (lane 3), and pUR250 (lane 2) by *Eco*RI digestion.

and the *Bam*HI restriction site at the coordinate at 21 kb in Tn*2424* (13, 31).

Tn*2424* is a transposable element of clinical and evolutionary significance. As has been discussed for transposable gentamicin resistance (10), serious epidemiological and therapeutic implications might arise because of the spread of Tn*2424* to different gram-negative species, like *Serratia* or *Pseudomonas*, especially as amikacin is the drug of last choice in the treatment of serious infections caused by multiresistant pathogenic bacteria. Nevertheless, dissemination of transposable amikacin resistance is restricted by the following circumstances: (i) generally gentamicin is used as the drug of first choice (group IV 6'-*N*-acetyltransferases do not mediate gentamicin resistance [Table 2]); (ii) the use of amikacin is restricted to the above-mentioned cases; and (iii) 6'-*N*-acetyltransferases do not always enable the producing organisms (e.g., *E. coli*) to withstand treatment with amikacin.

A second point of interest for Tn*2424* appears in view of the evolution of multiresistance tran-

sposons, as Tn*2424* may represent one final step in the phylogenetic sequence of related transposable elements.

De la Cruz and Grinsted (13) identified the close relationship between Tn*21* and Tn*501* (4) by comparing the *mer* genes, *tnpA* functions, and inverted repeats (38 base pairs [35]) of these transposons (transposon *Tn*501 has been the subject of previous work [1, 30]). By secondary rearrangement during the formation of cointegrates (13), Tn*21* may have evolved from an ancestral structure similar to Tn*501*. Furthermore, other Tn*21*-like structures have been described (17, 26, 33). However, despite the fact that these transposons include additional resistance genes, they are not larger than Tn*21* itself. Thus, only Tn*2424* includes the complete sequence of Tn*21* in addition to other DNA fragments.

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