Amikacin Resistance Mediated by Multiresistance Transposon Tn2424

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Tn2424, a multiresistance transposon 25 kilobases long, was isolated from IncFII plasmid NR79. Tn2424 transposed resistance to sulfonamides, streptomycin and spectinomycin, mercuric chloride, chloramphenicol, and amikacin with a frequency of $6 \times 10^{-5}$. 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 Tn2424. Comparison by a heteroduplex technique revealed that Tn2424 includes the total sequence of Tn21 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 Tn2424, 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 Tn2424 by restriction analysis and a hetero-duplex technique revealed its close relationship to Tn21, 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.
TABLE 1. Bacterial strains and plasmids used

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant markers*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli W677</td>
<td>F-, thr, leu, thi, lac, mal, gal</td>
<td>3</td>
</tr>
<tr>
<td>W3110</td>
<td>NaI', lac+</td>
<td>14</td>
</tr>
<tr>
<td>JC2926</td>
<td>Sm', recA, thi, thr, arg, his, leu, lac</td>
<td>2</td>
</tr>
<tr>
<td>SK1592</td>
<td>gal, thi, ton, sbcB15, hsr-4, hsm+</td>
<td>19</td>
</tr>
<tr>
<td>7118</td>
<td>Δ(lac-pro), F'lacPZΔM15 pro+</td>
<td>20</td>
</tr>
<tr>
<td>Proteus mirabilis Pm13</td>
<td></td>
<td>9</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa PAO303</td>
<td></td>
<td>24</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NR79</td>
<td>Tc', Su', Hg', Sm', Sp', Cm', Ami', Tra+</td>
<td>3</td>
</tr>
<tr>
<td>pUB307</td>
<td>Pm', Tc', Tra+</td>
<td>5</td>
</tr>
<tr>
<td>pBR322</td>
<td>Ap', Te'</td>
<td>6</td>
</tr>
<tr>
<td>pUR250</td>
<td>Ap'</td>
<td>29</td>
</tr>
<tr>
<td>pUB307::Tn2424</td>
<td>Pm', Tc', Su', Hg', Sm', Sp', Ami', Cm', Tra+</td>
<td>This paper</td>
</tr>
<tr>
<td>pBR322::Tn2424</td>
<td>Ap', Su', Hg', Sm', Ami', Cm'</td>
<td>This paper</td>
</tr>
<tr>
<td>pBR322::Tn21</td>
<td>Ap', Su', Hg', Sm', Te', Sp'</td>
<td>Kratzb</td>
</tr>
</tbody>
</table>

a NaI', Nalidixic acid resistance; Sm', streptomycin resistance; Rif', rifampin resistance; Tc', tetracycline resistance; Su', sulfonamide resistance; Hg', mercuric chloride resistance; Sp', spectinomycin resistance; Cm', chloramphenicol resistance; Ami', amikacin resistance; Pm', paromomycin resistance; Ap', 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 x 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-ß-D-thiogalactoside and 5-bromo-4-chloro-indolyl-ß-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 Tn2424. 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::Tn2424 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::Tn2424), with selection on M9-lactose minimal medium containing nalidixic acid, paromomycin, and amikacin. The resulting transconjugants [W3110(pUB307::Tn2424)] were tested for their resistance markers (Pm', Tc', Sp', Su', Hg', Ami', and Cm'). 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::Tn2424 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::Tn2424) was used for the experiments described below.

Transfer of Tn2424 from E. coli to Pseudomonas aeruginosa. To determine the transposition frequency of Tn2424, we transferred pBR322::Tn2424 from E. coli SK1592 to E. coli JC2926 (pUB307). E. coli JC2926 (pBR322::Tn2424 and pUB307) was mated with Pseudomonas aeruginosa PAO303. One-half of the Pseudomonas transconjugants were plated onto Cetrimid agar.
TABLE 2. Levels of resistance mediated by pUB307::Tn2424 in E. coli and Pseudomonas aeruginosa

<table>
<thead>
<tr>
<th>Drug</th>
<th>E. coli W3110</th>
<th>Pseudomonas aeruginosa PAO303</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No plasmid</td>
<td>pUB307::Tn2424</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>0.5</td>
<td>16</td>
</tr>
<tr>
<td>Sisomycin</td>
<td>0.5</td>
<td>4</td>
</tr>
<tr>
<td>Amikacin</td>
<td>0.5</td>
<td>16</td>
</tr>
</tbody>
</table>

*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 \(6 \times 10^{-5}\); this value was considered for the transposition frequency. The resulting *Pseudomonas* transconjugants mediated higher levels of resistance to 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 amino-glycoside-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 Tn2424. The plasmid DNA of strain SK1592(pBR322::Tn2424) was used for single and double digestions with the following restriction enzymes: EcoRI, BamHI, Sall, Smal, PstI, and BgIII. The restriction data resulted in a physical map of Tn2424 (Fig. 1). *EcoRI-BamHI digestion revealed that Tn2424 transposed into the *tet* gene adjacent to the *BamHI* 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 Tn2424.

**Analysis of Tn2424 by electron microscopy.** The physical map of Tn2424 led us to the conclusion that this transposable element is closely related to Tn21. Therefore, we determined the relationship between these two transposons by performing a heteroduplex analysis, using pBR322::Tn21 and pBR322::Tn2424 (Fig. 2A). As Tn21 and Tn2424 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 Tn21 is included in Tn2424, and, in addition, Tn2424 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 Tn2424 are inserted into EcoRI fragments K and G of Tn21. The DNA between the coordinates at 9.8 and 11.6 kb (Fig. 1) represents loop 1 and carries an additional *Smal* restriction site. The restriction sites for *Smal* (coordinate at 15 kb) and *EcoRI* (coordinate at 19 kb) flank nearly all of the sequence representing loop 2.

**Cloning experiments and functional map of Tn2424.** To correlate the *aac* and *cat* resistance genes of Tn2424 with the additional DNA segments (loops 1 and 2), we constructed a functional map of Tn2424 (Fig. 1). *EcoRI- and BamHI-digested DNA of pBR322::Tn2424 was cloned into pUR250 and used to transform *E. coli* 7118. White, ampicillin-resistant colonies **DIAGRAM**

FIG. 1. Physical and functional map of pBR322::Tn2424. The scale at the top represents the length of plasmid pBR322 (broken lines) and the length of Tn2424 (in kilobases). Tn2424 is inserted adjacent to the *BamHI* 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 Tn2424. 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*).
showing at least one additional resistance marker of Tn2424 were selected on Iso-Sensitest agar containing isopropyl-β-D-thiogalactoside and 5-bromo-4-chloro-indolyl-β-D-galactoside. Cloning experiments with EcoRI-digested DNA resulted in several Ap' Su' Cm' colonies, whereas other cloned resistance markers, such as amikacin, spectinomycin, and mercuric chloride, were not detected. Screening of 15 Ap' Su' Cm' colonies showed that EcoRI 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 pBR322::Tn2424 (in this case fragments E1 and E2) or EcoRI fragments E1 and the vector plasmid could be detected. The ampicillin resistance of clones containing ligated EcoRI fragments E1 and E2 was due to the presence of a part of pBR322 in fragment E2, as shown in Fig. 1. Cloning of BamHI-digested DNA resulted in Ap' Hg' and Ap' Ami' Sp' phenotypes. Besides the Ap' Hg' phenotype that was mediated by self-ligated BamHI fragment B1, we determined the fragment that was responsible for spectinomycin and amikacin resistance. Screening of several Ap' Ami' Sp' clones revealed that BamHI 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.

FIG. 2. (A) Heteroduplex formed by pBR322::Tn21 and pBR322::Tn2424. (B) Schematic drawing of the heteroduplex molecule. Bar = 0.5 μm. (C) Schematic structures of Tn21 (upper line) and Tn2424 (lower line), with their fragments generated by EcoRI digestion. The solid boxes at the ends of Tn21 and Tn2424 correspond to the inverted repeated ends of both elements. The nomenclature of the EcoRI fragments of TN21 has been described previously (7, 21).
We isolated Tn2424 from IncFII plasmid NR79, which affects the bactericidal activities of a great number of aminoglycosides, including kanamycin, tobramycin, as the result of Wiedemann, et al. (13). As Tn2424 includes the total sequence of Tn21, this finding supports the results of Haas and Davies (15), who showed the striking relationship between plasmids NR79 and R100.

The aac gene of Tn2424 is inserted together with the cat gene into EcoRI fragment G of Tn21, between the sul and aad genes (Fig. 2C). In this way the DNA segment carrying these additional resistance genes enlarges EcoRI fragment G by about 4 kb and leads to several additional restriction sites for SalI, PstI, BglII, EcoRI, and SmaI.

The failure to clone amikacin or spectinomycin resistance after EcoRI 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 EcoRI site that separates fragments E1 and E6 (Fig. 1). A second inserted DNA fragment (length, 1.8 kb) is located in EcoRI fragment K of Tn21, resulting in an additional SmaI restriction site. This fragment lacks detectable functions. The locations of other functional genes of Tn2424 were deduced from the homology between Tn2424 and Tn21 (Fig. 2). The mercury resistance gene has been located on EcoRI fragments E3 and E5 of Tn2424 (EcoRI 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 Tn2603 (31) and Tn21 (13). As Tn2603 has been shown to be closely related to Tn21 (33), we concluded that the transposase and resolvase are located within BamHI fragment B4 of Tn2424, corresponding to fragment B2 of Tn2603 (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 EcoRI fragment E8 and a part of fragment E2 in Tn2424. Resolvase (tnpR) seems to be encoded by the DNA fragment adjacent to the left side of the tnpA region and the BamHI restriction site at the coordinate at 21 kb in Tn2424 (13, 31).

Tn2424 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 Tn2424 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 Tn2424 appears in view of the evolution of multiresistance tran-
sponsons, as Tn2424 may represent one final step in the phylogenetic sequence of related transposable elements.

De la Cruz and Grinstead (13) identified the close relationship between Tn21 and Tn501 (4) by comparing the mer genes, tnpA functions, and inverted repeats (38 base pairs [35]) of these transposons (transposon Tn501 has been the subject of previous work [1, 30]). By secondary rearrangement during the formation of cointegrates (13), Tn21 may have evolved from an ancestral structure similar to Tn501. Furthermore, other Tn21-like structures have been described (17, 26, 33). However, despite the fact that these transposons include additional resistance genes, they are not larger than Tn21 itself. Thus, only Tn2424 includes the complete sequence of Tn21 in addition to other DNA fragments.

LITERATURE CITED


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