Identification of Tn2401, a Transposon Encoding Multiresistance to Aminoglycosides

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A transposable element, Tn2401, was found in a clinical isolate of *Pseudomonas aeruginosa*. Tn2401 had a size of 7190 nucleotides and encoded aminoglycoside 3′-phosphotransferase and aminoglycoside 6′-*N*-acetyltransferase. The sequence encoding the former enzyme was homologous with that of Tn903. *Pseudomonas aeruginosa* strains harbouring this transposon were resistant to kanamycin, neomycin, lividomycin, ribostamycin, paromomycin, netilmycin, tobramycin, dibekacin, gentamicin, sisomicin, and butirosin.

INTRODUCTION

Aminoglycoside antibiotics such as gentamicin, tobramycin, sisomicin, dibekacin, and amikacin are still of great importance in the treatment of life-threatening infections, but there are many reports of serious infections caused by aminoglycoside-resistant *Enterobacteriaceae* and *Pseudomonas* strains in hospitals (Acar *et al.*, 1977; Yoshikawa *et al.*, 1978). In clinical isolates resistance to aminoglycoside–aminocyclitol antibiotics is usually associated with the presence of enzymes that catalyse *N*-acetylation, *O*-phosphorylation, or *O*-adenylylation of amino or hydroxyl groups on the drug molecule (Beneviste & Davies, 1973). Some of these aminoglycoside modifying enzymes are encoded by genes capable of moving from one replicon to another in the absence of host-mediated recombination functions. Such movable genetic elements have been termed transposons (Starlinger, 1980). Two of them, Tn903 and Tn5, encode aminoglycoside 3′-phosphotransferases APH-(3′)I and APH-(3′)II, respectively, which phosphorylate kanamycin, neomycin, and related compounds (Yamamoto & Yokota, 1980). Nugent *et al.* (1979) identified Tn732, the first transposon mediating multiple resistance to the more 'modern' aminoglycosides such as gentamicin, tobramycin, sisomicin, and dibekacin by the production of aminoglycoside 2″-adenylyltransferase [ANT-(2″)].

In this paper we report the identification of a transposon coding for the production of two inactivating enzymes: the aminoglycoside 3′-phosphotransferase, APH-(3′)I, which mediates resistance to kanamycin, neomycin, paromomycin, ribostamycin, and lividomycin; and the aminoglycoside 6′-*N*-acetyltransferase, AAC-(6′), which modifies gentamicin, tobramycin, sisomicin, kanamycin, neomycin, dibekacin, butirosin, and netilmycin.

Abbreviations: AAC-(6′), aminoglycoside 6′-*N*-acetyltransferase (EC 2.3.1.55); ANT-(2″), aminoglycoside 2″-adenylyltransferase (EC 2.7.7.46); APH-(3′)I, aminoglycoside 3′-phosphotransferase (EC 2.7.1.95); APH-(6), aminoglycoside 6-phosphotransferase (EC 2.7.1.72); Ap, ampicillin; But, butirosin; Cm, chloramphenicol; Dk, dibekacin; Gm, gentamicin; Km, kanamycin; Liv, lividomycin; Nal, nalidixic acid; Net, netilmycin; Nm, neomycin; Pm, paromomycin; Rif, rifampicin; Rm, ribostamycin; Sis, sisomicin; Sm, streptomycin; Su, sulphonamides; Tc, tetracycline; Tm, tobramycin.

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METHODS

Bacterial strains and plasmids. These are listed in Table 1.

Culture conditions. Growth media and testing of antibiotic sensitivity were as described recently (van Treeck *et al.*, 1981).

Plasmid techniques. Preparation of plasmid DNA, restriction enzyme analysis, conjugational transfer and transformation of DNA were carried out as described previously (Schmidt *et al.*, 1982).

Measurement of enzyme activities. Substrate activities of aminoglycoside modifying enzymes were measured according to the paper binding method (Beneviste & Davies, 1971).

Transposition experiments and phage techniques. Transposition of aminoglycoside resistance markers to P1CmC1$_{ts}$, preparation of phage lysates by heat induction, phage titration, tests for phage production, plaque centre tests, determination of phage immunity and transduction were done as described by Iida & Arber (1977). Curing of phage lysogenic cells was done as described by Rosner (1972), with the addition of 5 mM-citrate to prevent phage reinfection.

Heteroduplex analysis. This was performed according to the formamide technique of Davis *et al.* (1971). For length calculations, double and single stranded DNA of phage ϕX174 was used as an internal standard, giving a length of 5375 bp (Sanger *et al.*, 1977). The contour length of DNA was measured with a Numonics digitizer.

Hybridization of DNA separated on agarose gels. The method of Southern (1975) as adapted to DNA–DNA hybridization by Botchan *et al.* (1976) was used with minor modifications (Wahl *et al.*, 1979). P1Cm$_{ts}$ DNA was nick-translated as described by Maniatis *et al.* (1975).

RESULTS

Transposition of aminoglycoside genes on to various phages and plasmids

Plasmid pBP30, coding for the production of APH-(3′)I, APH-(6), and AAC-(6′), was originally found in *Pseudomonas aeruginosa* 141, which was isolated from a clinical specimen at the University Hospital, Bonn. pBP30 is a nonconjugative plasmid of 43·2 kb and confers resistance only to aminoglycoside antibiotics (Table 1). Resistance to aminoglycosides mediated by the AAC-(6′) enzyme (gentamicin, tobramycin, dibekacin, sisomicin, netilmycin and butirosin, see Table 1) is only weakly expressed in *Escherichia coli* hosts, as could be demonstrated by transfer of pBP30 DNA according to the method of Sinclair & Morgan (1978). Plasmid pBP30 DNA was isolated from *P. aeruginosa* 141, transformed to *E. coli* and back to *P. aeruginosa* (Wiedemann & Weppelmann, 1981). *Escherichia coli* transformants produced all three transferases, but were resistant only to kanamycin, neomycin, paromomycin, lividomycin, streptomycin, tobramycin, and dibekacin.

By the use of the method of Iida & Arber (1977), a potential transposon was identified from pBP30 with the aid of bacteriophage P1Cm$_{ts}$. Plasmid DNA from *E. coli* strain SK1592(pBP30) was transformed into *E. coli* C600 Sm^r *recA* that was lysogenic for phage P1Cm$_{ts}$. Phage lysates were prepared by heat induction (Iida & Arber, 1977) and used to infect *E. coli* WA921. Kanamycin-resistant clones were selected at 30 °C, and checked for resistance to tobramycin. A total of 15 strains were selected and phages were checked by plaque centre testing (Iida & Arber, 1977) for resistance to chloramphenicol, kanamycin, and tobramycin. Two P1Cm$_{ts}$ Km^r Tm^r lysogenic strains were isolated, induced to prepare phage lysates, and 49 single plaques were again tested by the plaque centre test. Of 49 plaques, 43 contained P1Cm$_{ts}$ Km^r Tm^r phages. The resistance patterns of the clones lysogenic for transposon-bearing phages demonstrated that the sequence transposed to P1Cm$_{ts}$ carried the genes for two of the aminoglycoside modifying enzymes, APH-(3′)I and AAC-(6′). In agreement with the Plasmid Reference Center this transposon has been designated Tn2401 (Lederberg, 1981).

In order to determine the physical characteristics of the transposable element, *E. coli* JC2926 *recA* carrying pBR322 was infected with P1Cm$_{ts}$ Km^r Tm^r phages at a multiplicity of 1 p.f.u. per cell. Lysogens were selected on ampicillin plus kanamycin medium at 30 °C and tested for resistance to ampicillin, tetracycline, chloramphenicol, kanamycin and tobramycin, and for phage production. Six strains were isolated and cured for bacteriophage production by incubation of diluted overnight cultures on ampicillin plus kanamycin medium at 42 °C (Rosner, 1972). After incubation for 24 h at 42 °C, survivors were further purified on ampicillin plus kanamycin medium at 42 °C and tested for chloramphenicol sensitivity, phage sensitivity,

Table 1. *Bacterial strains, plasmids and phages*

Bacterial strains	Relevant characteristics	Source or reference
E. coli WA921 Nal^r	*lac thr leu met thi hsd*	Wood (1966)
E. coli SK1592	*gal thi* $T1^r$ *sbcB15 hsdR4 hsdM endA*	Kushner (1978)
E. coli JC2926 Rif^r	*recA thi thr arg his leu mal*	Bachmann (1972)
E. coli C600 Sm^r	*recA thr leu thi tonA*	Cohen *et al.* (1972)
P. aeruginosa 2029 Rif^r	*leu res*	A. M. Chakrabarty
Plasmids		
pBP30	Sm^r Km^r Nm^r Pm^r Liv^r Rm^r Tm^r Dk^r Gm^r Net^r Sis^r But^r	Wiedemann & Weppelmann (1981)
pBR322	Ap^r Tc^r	Bolivar *et al.* (1977)
RSF1010	Sm^r Su^r	Guerry *et al.* (1974)
pSC105	Km^r Tc^r	Nisen *et al.* (1977)
R64-11	Mutant of R64; Tc^r Km^r	Meynell & Datta (1976)
pBP301, pBP302, pBP303, pBP304	Ap^r *aphA* (Km^r Nm^r Pm^r Liv^r Rm^r) *aacA* (Tm^r Dk^r Gm^r Net^r Sis^r But^r)	This paper; pBR322::Tn2401
pB305	Sm^r Km^r Nm^r Pm^r Liv^r Rm^r Tm^r Dk^r Gm^r Net^r Sis^r But^r	This paper; *Pst*I fragment C of pBP301 inserted in RSF1010
Phages		
$P1Cm_{ts}$	Heat inducible; Cm^r	Mise *et al.* (1976)
$P1Cm_{ts}$	Heat inducible; Cm^r Km^r Nm^r Pm^r Liv^r Rm^r Tm^r Dk^r Gm^r Net^r Sis^r But^r	This paper

bacteriophage production and resistance to ampicillin, tetracycline, kanamycin, and tobramycin (Rosner, 1972).

Four clones which produced no bacteriophages (i.e. cured cells) were picked and further characterized. They exhibited resistance to ampicillin, kanamycin, and tobramycin but were sensitive to tetracycline.

Cell-free extracts of the four strains carrying pBR322::Tn2401 were tested for the presence of aminoglycoside modifying enzymes. Besides the MIC values, the results of these assays (Table 2) clearly showed that the chimaeric plasmids produced APH-(3′)I and AAC-(6′). Resistance to streptomycin or inactivation of that drug did not occur. However, when bacteria bearing pBR322::Tn2401 were subjected to osmotic shock (Beneviste & Davies, 1971), the cell-free supernatants had definite but lower levels of AAC-(6′) than those of *P. aeruginosa* 141. When the DNA segment of pBP301 (pBR322::Tn2401), containing the AAC-(6′) gene (*aacA*; *Pst*I fragment C, see also below) was cloned into RSF1010, and the resulting chimaeric plasmid, pBP305, was transferred to *P. aeruginosa* 2029, these cells contained 100% more AAC-(6′) than similar preparations from SK1592 harbouring pBP301. The observation that *aacA* is only weakly expressed in *E. coli* strains cannot be explained from our experimental approach.

From agarose gel electrophoresis, hybrid plasmids pBR322::Tn2401, prepared from all four clones had a size of 11·5 kb, indicating the transposition of a 7·1 kb DNA sequence on to pBR322. The purified DNAs of these plasmids, pBP301, pBP302, pBP303, and pBP304, were compared by digestion with restriction endonuclease *Bgl*I. They all yielded 12 fragments (Table 3), forming identical restriction patterns in agarose slab gels, indicating that the aminoglycoside resistance genes had transposed to the same site in pBR322, i.e. the Tc^r gene, corresponding with the loss of tetracycline resistance.

To exclude the possibility that sequences from pBP30 had been picked by a *recA*-independent process directed by the IS1 elements or other sequences in $P1Cm_{ts}$, DNA sequence homology between phage DNA and restriction fragments of the hybrid plasmids was tested by Southern

Table 2. *Acetylation, phosphorylation and MIC values of aminoglycosides by Tn2401-encoded transferases AAC-(6′) and APH-(3′)I*

Abbreviations: A, acetylation, expressed as percentage of activity against kanamycin A substrate as determined by radioassay; B, phosphorylation, expressed as percentage of activity against lividomycin A substrate as determined by radioassay; C, MIC values (μg ml^{-1}). The control values for acetylation of kanamycin A were: *P. aeruginosa* 141, 5800 c.p.m.; *E. coli* SK1592(pBP30), 2201 c.p.m.; *E. coli* Sk1592(pBP301), 1386 c.p.m.; *P. aeruginosa* 2029(pBP305), 3482 c.p.m. The control values for phosphorylation of lividomycin A were: *P. aeruginosa* 141, 3582 c.p.m.; *E. coli* SK1592(pBP30), 3188 c.p.m.; *E. coli* SK1592(pBP301), 3092 c.p.m.; *P. aeruginosa* 2029(pBP305), 3704 c.p.m.

	P. aeruginosa 141 (pBP30)			*E. coli* SK1592 (pBP30)			*E. coli* SK1592 (pBP301)			*P. aeruginosa* 2029 (pBP305)		
	A	B	C	A	B	C	A	B	C	A	B	C
Kanamycin A	100	35	256*	100	32	256*	100	30	256*	100	32	256*
Tobramycin	69	—	64	88	—	8	79	—	4	80	—	16
Sisomicin	95	—	128	53	—	2	59	—	2	61	—	2
Dibekacin	93	—	128	78	—	4	66	—	4	72	—	8
Gentamicin $C_{1\alpha}$	70	—	32	58	—	1	60	—	1	56	—	2
Neomycin	49	69	128*	23	62	128*	21	57	64*	25	58	128*
Lividomycin A	—	100	1000	—	100	1000	—	100	1000	—	100	1000
Paromomycin	—	82	1000	—	84	1000	—	85	1000	—	81	1000

* These values are due to the action of APH-(3′)I.

Table 3. *Restriction fragments of pBP301, pBP302, pBP303, and pBP304*

Plasmid DNAs were digested with restriction enzymes and the resulting DNA fragments were separated by electrophoresis in 0·8 or 1·2% (w/v) agarose slab gels (25 × 14 × 0·4 cm) for 15 h at 3 V cm^{-1}. λ DNA treated with either *Eco*RI, *Hin*dIII, or *Pst*I was used to provide size markers for digested plasmid DNA.

	Size (kb)										
Fragment	*Eco*RI	*Sma*I	*Xho*I	*Sal*I	*Hin*dII	*Hin*dIII	*Pst*I	*Bgl*I	*Xho*I + *Sma*I	*Xho*I + *Hin*dIII	*Sma*I + *Hin*dIII
A	11·55	11·55	9·75	5·61	5·61	8·1	4·85	2·4	9·65	6·05	7·9
B			1·85	5·15	3·2	2·45	2·7	2·0	1·85	2·45	2·45
C				0·81	1·93	0·85	2·45	1·6	0·26	1·85	0·85
D					0·81		1·65	1·2		0·85	0·22
E								0·9		0·49	
F								0·7			
G								0·57			
H								0·34*			
I								0·3			
J								0·23*			

* The corresponding bands in the restriction pattern contain two fragments of the same size.

hybridization (Southern, 1975). DNA of one of the chimaeric plasmids, pBP301, was digested with *Hin*dIII and *Pst*I, and $P1Cm_{ts}$ DNA with *Pst*I. The fragments were then separated by gel electrophoresis. After transfer from the gel to a nitrocellulose filter they were subjected to Southern hybridization with ^{32}P-labelled $P1Cm_{ts}$ DNA. *Pst*I fragments of phage DNA served as control and could be visualized after a few hours of autoradiography. Even after a three week exposure of the autoradiogram, no sequence homology between Tn2401 and phage DNA was detected. Thus, chromosomal aberrations, as have been described for cointegrates between P1 and the r-determinant of NR1 (Iida & Arber, 1980), did not seem to direct transposition of Tn2401.

To confirm the transposition ability of Tn2401 in the absence of a functional *recA* gene, the

element was transposed to plasmid R64-11 (Meynell & Datta, 1976) which codes for resistance to streptomycin and tetracycline. R64-11 was transferred to *E. coli* K12 *recA* (pBR322::Tn2401) by conjugation and then to *E. coli* WA921 with selection for kanamycin resistance. All transconjugants were sensitive to ampicillin but resistant to kanamycin, indicating that pBR322::Tn2401 had not been mobilized while the element Tn2401 had been transposed to R64-11 prior to conjugational transfer. All the exconjugants had lost resistance to either streptomycin or tetracycline. Three tetracycline-sensitive clones and two streptomycin-sensitive clones were further analysed. The MIC values for kanamycin (256 μg ml^{-1}), neomycin (64 μg ml^{-1}), paromomycin (1 μg ml^{-1}), tobramycin (4 μg ml^{-1}), dibekacin (4 μg ml^{-1}), and tetracycline (256 μg ml^{-1}) or streptomycin (256 μg ml^{-1}) agreed with the corresponding values for K12 *recA* (R64-11) (pBR322::Tn2401). Analysis of the plasmid DNA from these strains by gel electrophoresis and electron microscopy demonstrated that there were no replicons of the same size as pBP301. Plasmid R64-11 containing Tn2401 was transferred by conjugation with the same frequency as found for R64-11 (10^{-1}). We concluded that Tn2401 transposed to different sites in R64-11.

Sequence homology with Tn903

As reported by Oka *et al.* (1981) Tn903 codes for a phosphotransferase, APH-(3′)I. The resistance gene of the element is flanked by large inverted repeats (IS903) 1057 bp in length. Both the structural gene and the insertion element carry characteristic restriction sites: IS903 is cleaved twice with *Sma*I, and the APH-(3′)I gene (*aphA*) once with *Xho*I, *Sma*I, and *Hin*dIII. When cleaved with combinations of the three restriction enzymes, pBP301 yielded the same fragments found in the structural gene of Tn903 in pSC105, but lacked the fragments produced by cleavage of IS903. Sequence analysis of Tn903 (Oka *et al.*, 1981) demonstrated a length of 276 bp for the *Xho*I–*Sma*I fragment found in *aphA*, 520 bp for the *Xho*I–*Hin*dIII fragment, and 244 bp for the *Sma*I–*Hin*dIII fragment which resembled the corresponding fragments of Tn2401; the latter were calculated to be 260 bp, 490 bp and 220 bp, respectively (Table 3). Figure 1 shows a heteroduplex structure formed between pSC105, which contains Tn903, and pBP301. An 840 ± 35 nucleotide-long region of DNA homology was found in the loop segments of both transposable elements. This homology is in good agreement with the length of *aphA* (813 nucleotides) reported from the sequence analysis of Tn903 (Oka *et al.*, 1981).

Genetic and physical map of pBP301

To locate the integration site of Tn2401 in pBR322, a heteroduplex between pBR322 and pBP301 was made and analysed by electron microscopy. The single *Eco*RI site of pBR322 was used as an internal reference point. A total of 21 heteroduplex structures demonstrated a linear double stranded region with the size of pBR322 DNA and a single stranded loop corresponding to a 7·19 ± 0·33 kb sequence (Tn2401) which was 0·34 kb from the *Eco*RI site. A short double stranded 'stalk' flanked the loop, representing inverted repeats (Fig. 2). Comparative measurements of the inverted repeats flanking Tn2401 and Tn3 (38 nucleotides; Ohtsubo *et al.*, 1978) revealed the same order of magnitude for both duplications. The loop segment in the heteroduplex molecule also exhibited an internal hairpin loop structure. A total of 25 of the internal hairpin loops were measured. The stem region was calculated to be 0·22 ± 0·02 kb and the loop region to be 1·82 ± 0·07 kb (Fig. 2).

Plasmid pBP301 was digested with seven different restriction endonucleases (Table 3), singly or in appropriate combinations. After electrophoresis the fragment sizes were calculated. In addition to the restriction analysis, *Hin*dIII digests of pBR322 (one fragment) and pBP301 (three fragments) were subjected to heteroduplex analysis and the length of the corresponding structures calculated. From these data a cleavage map of pBP301 could be constructed. By comparison with the heteroduplex structures formed between Tn903 and Tn2401 (Fig. 1), the APH-(3′)I gene was located on the circular restriction map of pBP301 (Fig. 3).

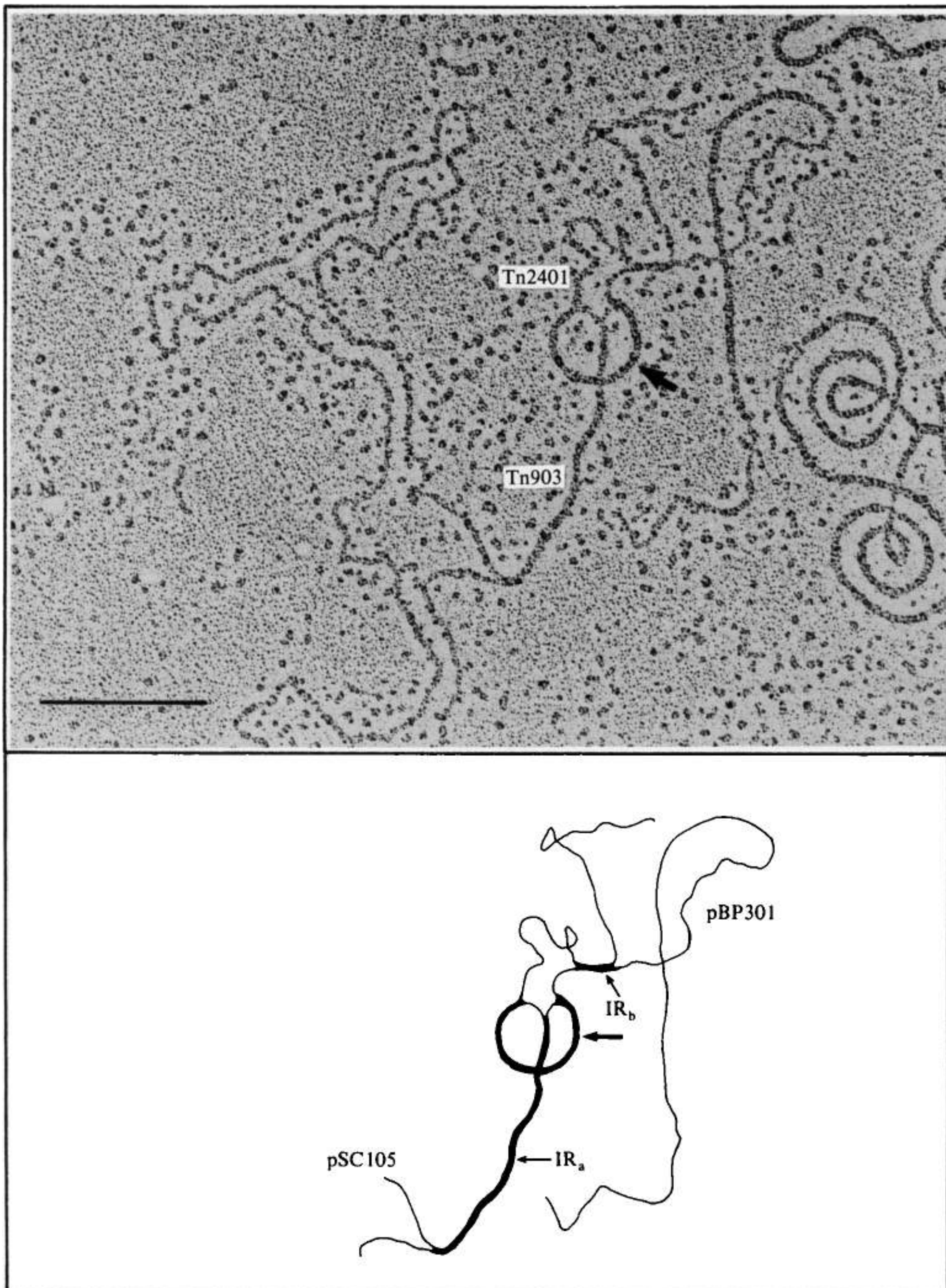

Fig. 1. Heteroduplex formation between pSC105 and pBP301. The region of homology formed between the loop structures of Tn2401 and Tn903 is indicated by a bold arrow. Inverted repeat (IR) sequences are indicated by fine arrows. IR_a represents IS903, and IR_b represents the 0·22 kb segment in pBP301. The bar marker represents 0·2 μm.

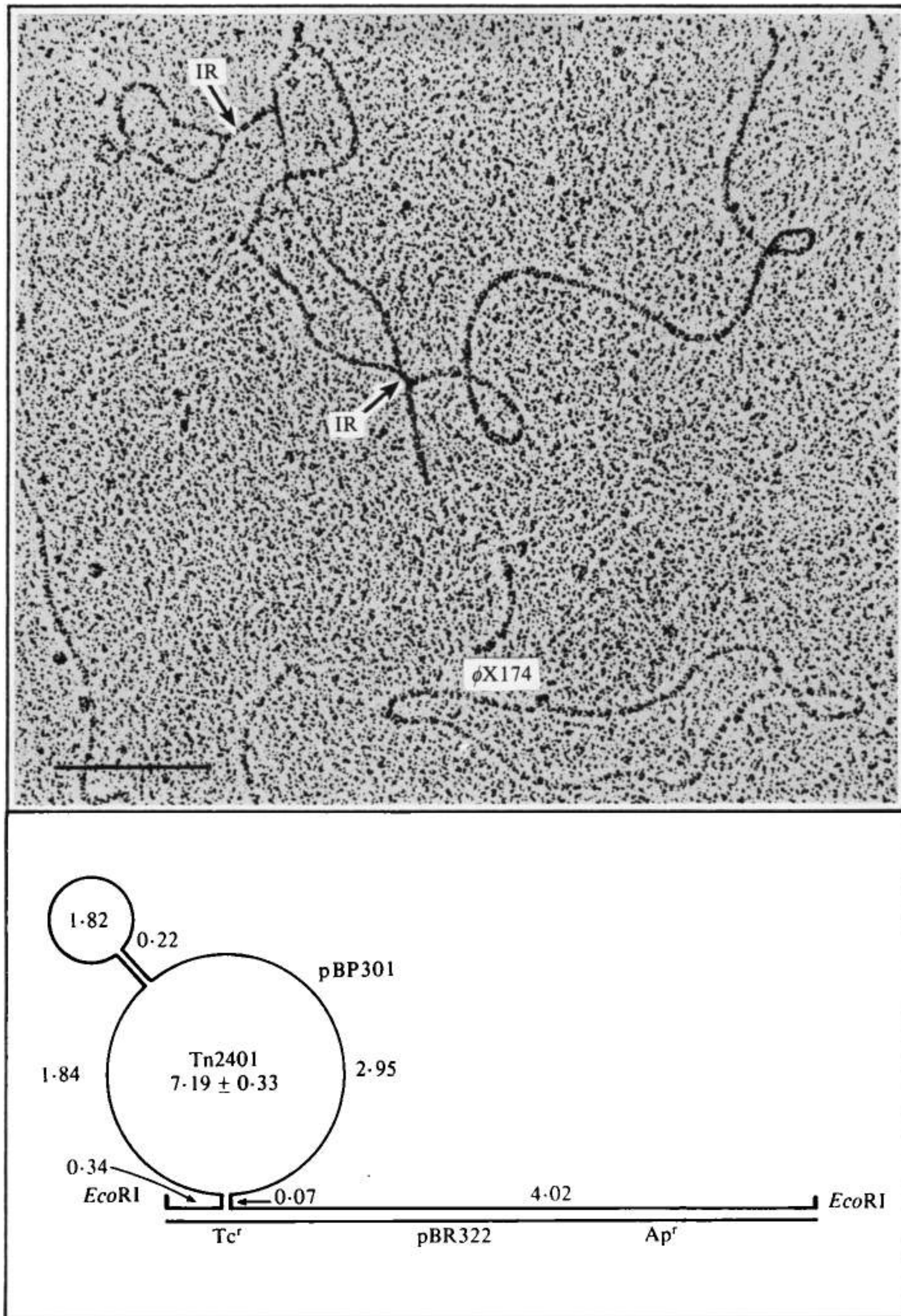

Fig. 2. Heteroduplex formation between pBR322 and pBP301. Both plasmids were digested with *Eco*RI to give linear molecules prior to denaturation and renaturation of DNA. The position of the two different inverted repeats (IR) are indicated by arrows in the micrograph. Single stranded ϕX174 DNA is also indicated. The bar marker represents 0·2 μm.

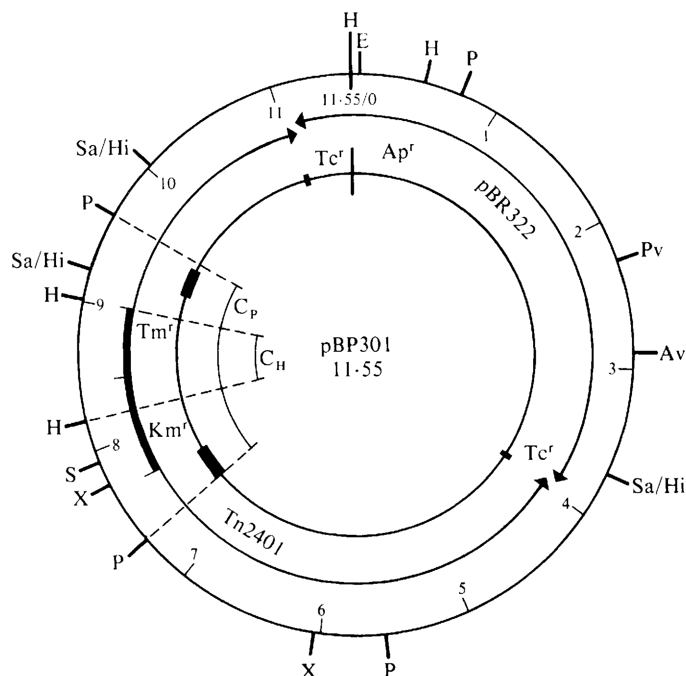

Fig. 3. Circular physical and functional map of pBP301 (pBR322::Tn2401). Coordinates are given in kb relative to the single *Hin*dIII site in pBR322. The small black boxes indicate the position of the inverted repeats. The large box represents the position of *aphA* (Km^r) and *aacA* (Tm^r). Restriction fragments used to localize the resistance genes by cloning procedures are designated as follows: C_H, *Hin*dIII fragment C; and C_P, *Pst*I fragment C (Table 3). Restriction sites: H, *Hin*dIII; E, *Eco*RI; P, *Pst*I; Pv, *Pvu*II; Hi, *Hin*dII; Sa, *Sal*I; S, *Sma*I; X, *Xho*I; Av, *Ava*I. The extent of pBR322 and Tn2401 is demonstrated by arrows. The physical data of pBR322 are from Sutcliffe (1978).

Cloning and localization of the AAC-(6′) gene

*Pst*I-generated fragments of pBP301 were cloned into the wide host range plasmid RSF1010. *Pst*I cleaved RSF1010 twice, yielding a small fragment covering the functions for replication and the streptomycin resistance gene. Ligated fragments were transformed into *E. coli* SK1592 and the resulting clones selected on tobramycin and paromomycin, the antibiotics inactivated by AAC-(6′) and APH-(3′)I, respectively. All the clones that were resistant to tobramycin, paromomycin and streptomycin but sensitive to sulphonamides contained *Pst*I fragment C integrated into RSF1010 (see Fig. 3). This result was confirmed and further defined by deletion of *Hin*dIII fragments from pBP301 and subsequent transformation of the resulting plasmids. Analysis of the corresponding clones revealed that the deletion of the smallest *Hin*dIII fragment, C, resulted in complete sensitivity to all aminoglycosides, while antibiotic susceptibility was not influenced by loss of fragment B. On the basis of these data *aacA* could be localized next to the *aphA* (Fig. 3).

Restriction map of the aminoglycoside resistance region of Tn2401

To construct a fine structural map of the resistance determinant segment of Tn2401, suitable restriction fragments from pBP301 were separated by electrophoresis on Sea-plaque agarose (FMC, Rockland, Me 04841, U.S.A.), isolated, purified and subjected to further restriction analysis on polyacrylamide gels. *Hin*dIII fragment C, which is known to contain part of *aphA* and *aacC*, and *Pst*I fragment C, containing both resistance genes, were purified and digested with restriction enzymes *Sau*3A, *Bgl*I, *Bgl*II, *Hpa*II, *Sal*I, or *Alu*I. The relative locations of the cleavage sites were determined by digestion, using restriction sites on Tn903 (Oka *et al.*, 1981) as

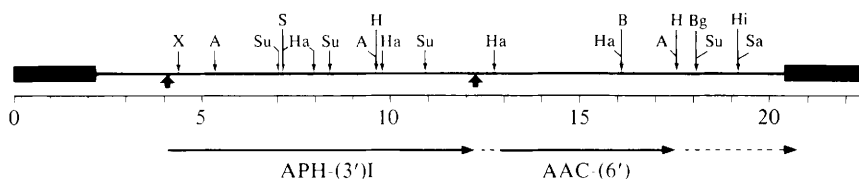

Fig. 4. Physical and functional map of the resistance region of Tn2401. Black boxes indicate inverted repeats. Coordinates are given in 100 bp units. The extent of homology to Tn903 is within the bold arrows. The coding regions of APH-(3′)I and AAC-(6′) are represented by continuous lines; broken lines represent the possible extent of the AAC-(6′) coding region (see text). The APH-(3′)I map is from Oka *et al.* (1981). Restriction sites: X, *Xho*I; H, *Hin*dIII; S, *Sma*I; B, *Bgl*I; Bg, *Bgl*II; A, *Alu*I; Sa, *Sal*I; Hi, *Hin*dII; Su, *Sau*3A; Ha, *Hpa*II. Three *Hpa*II fragments in the *Bgl*I–*Hin*dII fragment are not indicated, since their order remains unclear.

reference points. The cleavage map derived from these data and the heteroduplex analysis between Tn903 and Tn2401 (Fig. 1) is shown in Fig. 4.

DISCUSSION

We report the isolation of a new transposable element, Tn2401, coding for the production of two aminoglycoside inactivating transferases, AAC-(6′) and APH-(3′)I. In *P. aeruginosa*, resistance to gentamicin, tobramycin, sisomicin, netilmycin, and dibekacin is mediated by *aacA* of Tn2401, while resistance to paromomycin, lividomycin, kanamycin, and neomycin is due to the phosphotransferase, APH-(3′)I. As shown by heteroduplex mapping and restriction analysis, the APH-(3′)I genes of Tn2401 and Tn903 are homologous. As no additional homologous sequences were detected, the remaining parts of the transposons appear to have originated from different sources.

As deduced from the cloning experiments, the length of *aacA* is between 0·6 and 0·8 kb. The exact length of the gene remains unclear, since neither the control region nor the terminating codon is known. The 0·22 kb inverted repeats on either side of the resistance genes in Tn2401 are interesting, but their function is not yet understood.

Four independent clones harbouring pBR322::Tn2401 were isolated. A comparison of their restriction patterns indicates that the transposable element might have inserted into a specific site in pBR322. Additional experimental data are required to determine if the integration of Tn2401 is site-specific, a phenomenon which has been reported for only a few transposable elements, such as Tn554 (Krolewski *et al.*, 1981) and Tn7 (Lichtenstein & Brenner, 1982). Tn7 transposes to other sites when the attachment site is not available, a process which might be compared with the integration of Tn2401 in R64-11. Until these integration sites on R64-11 have been mapped or sequenced, it cannot be deduced whether multiple sites or only two preferred sites are available in R64-11 for integration of Tn2401 – one in the Sm^r gene and one in the Tc^r gene. The latter may be the same as the integration sites in pBR322.

Exact physical data about the characteristics of the target sequence and mutants defective in transposition will help to understand the nature of Tn2401 and the way genes encoding AAC-(6′) disseminate in Gram-negative hosts.

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