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Structure and Mobilization of an Ampicillin and Gentamicin Resistance Determinant

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A DNA segment originally found in an epidemic plasmid of *Escherichia coli* encoding an aminoglycoside-(3)-*N*-acetyltransferase gene (*aacC5*) and a TEM-type β-lactamase gene was characterized. The two genes were adjacent and constituted a single transcriptional unit. In addition, these genes were simultaneously mobilized through the action of an insertion sequence related to IS*26*, IS*140*, and IS*15*-Δ. This DNA segment is a composite transposon which has been called Tn*2922*.

Gentamicin resistance (Gm^r) and ampicillin resistance (Ap^r) genes have always been found together in plasmids isolated at the Hospital Clinico Universitario of Zaragoza (8, 19). Some of these plasmids have been characterized previously. For instance, a large (90-kilobase [kb]) conjugative plasmid belonging to incompatibility group M was often isolated from different enterobacterial genera. Its gentamicin resistance gene was identified as *aacC5*. This gene encodes the synthesis of an aminoglycoside-(3)-*N*-acetyltransferase of class V (9). Its resistance to ampicillin was mediated by TEM-1 β-lactamase.

In the course of these studies, the loss of a plasmid was sometimes observed without the simultaneous loss of its phenotypic characters. *Escherichia coli* 3644 is a J62 derivative that probably arose by insertion of one of these epidemic plasmids (pUZ3644) into the chromosome. This strain showed the resistance markers of pUZ3644 but did not contain plasmid DNA. This strain was used to study the mobilization and structure of the Gm^r and Ap^r genes present in those plasmids. As a result of this study, the *aacC5* gene has been identified and found to be related to the *aacC3* gene (1), the genes being distinguished by the substrate specificity of their products. The *bla* gene was found to be contained in a Tn*3*-like structure. However, this is the first time that *aacC5* has been described in a transposable element. The mobility of this element resides in two directly repeated copies of an insertion sequence (IS) beloning to the well-known IS family that includes IS*26* (17), IS*140* (3, 4), and IS*15*-Δ (21).

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used in this study are shown in Table 1.

Media and antibiotics. L broth and L agar were used throughout. The usual drug concentrations were as follows: ampicillin, 100 μg/ml; gentamicin, 20 μg/ml; streptomycin, 20 μg/ml; and spectinomycin, 20 μg/ml. To select cloned fragments in pUC8, we supplemented L agar plates with 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside per ml and 0.2 mmol of isopropyl-β-D-thiogalactopyranoside per liter. This allowed us to distinguish between recombinant- and vector-containing colonies (22). Antibiotic susceptibility testing was carried out by the disk diffusion method on Mueller-Hinton agar plates.

Genetic techniques. Conjugation was carried out on nitrocellulose membrane filters (0.22-μm pore size; Millipore Corp.) (2). Transformations were performed by the method of Cohen et al. (7). Transposition was assayed by mobilization of the resistance markers (5) by conjugative plasmid pUZ8 (Table 1), which is devoid of any known transposable element (23).

Plasmid DNA preparation and analysis. To test for the presence of plasmids, we prepared DNA from 10-ml cultures by the method of Ish-Horowicz and Burke (12). For cloning, sequencing, and electron microscopy, plasmid DNA was prepared from cleared lysates by cesium chloride-ethidium bromide density gradient centrifugation (6). Electrophoresis of plasmid DNA molecules and restriction fragments was performed as described by Meyers et al. (16).

DNA enzymes. Restriction endonucleases were purchased from Amersham Corp., Boehringer Mannheim Biochemicals, and New England BioLabs, Inc. T4 DNA ligase was purchased from New England BioLabs. *E. coli* alkaline phosphatase and *E. coli* DNA polymerase I (Klenow fragment) were purchased from Boehringer Mannheim Biochemicals. All enzymes were used in accordance with the instructions of the suppliers.

Electron microscopy. For homoduplex experiments, plasmid DNA was linearized with *Xba*I, denatured with alkali, neutralized, and allowed to renature for 5 min at 37°C in 50% formamide. Renatured DNA was prepared by the monolayer method of Kleinschmidt (13), shadowed with platinum, and examined and photographed in a Zeiss electron microscope. Measurements were made with the Videoplan (Kontron) image analysis system.

Sequence determination. DNA sequences were determined by the chemical degradation method of Maxam and Gilbert (15). Selected restriction fragments were cloned in vector pUC8 (22) and sequenced (see Fig. 3).

Insertion mutagenesis. The 2-kb mutational insertion Ω fragment encoding streptomycin resistance (Sm^r) and spectinomycin resistance (Sp^r) was prepared from plasmid pHP45 (Table 1) by digestion with *Sma*I. This DNA fragment contains transcription terminators at both ends, causing polar mutations at the point of insertion (18). Plasmid

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TABLE 1. Bacterial strains and plasmids[a]

Strain or plasmid	Relevant genotype or phenotypic markers	Size (kb)	Source or reference
Strains			
3644	Wild type		This study
J62	F⁻ *his lac* Nal^r *pro trp*		8
J53	F⁻ *met pro* Rif^r		8
AB2463	F⁻ *ara-14 argE galK2 his lacY1 leu proA recA rpsC thi thr tsx xyl*		24
UB5201	F⁻ *met (gyrA) nalA pro recA56*		24
SU200	F⁻ *leu recA trp*		Our laboratory collection
Plasmids			
pACYC184	Cm Tc Tra⁻ Mob⁻	4.1	20
pUZ8	Hg Km Tc Tra⁺	52	8, 23
pUC8	Ap Mob⁻ Tra⁻	2.7	22
pHP45	Ap Sm Sp Tra⁻ Mob⁻	4.3	18
pUZ3644	Ap Cm Gm Sm Su Tc Tra⁺	160	This study
pUZ999	Ap Cm Gm Hg Km Tra⁺	79	This study
pUZ1000	Ap Cm Gm Tra⁻	31	This study
pUZ1001	Ap Cm Gm Tra⁻	12.2	This study
pUZ1002	Cm Tra⁻	5	This study
pUZ1003	Ap Cm Gm Tra⁻	8.4	This study
pUZ1004	Ap Cm Gm Tra⁻	7	This study
pUZ1005	Ap Cm Gm Tra⁻	12.2	This study

[a] All strains are *E. coli* derivatives. Tra, Transfer proficiency; Mob, mobilization proficiency.

pUZ1004 was partially digested with either *Hae*III or *Bst*EII to generate mainly linear forms. The insertion fragment DNA and linearized pUZ1004 DNA were mixed and incubated with T4 DNA ligase. The ligation mixture was used to transform competent *E. coli* SU200 cells to Sm^r Sp^r.

RESULTS

Transposability of Ap^r and Gm^r genes. From 1979 to 1983, strains of gram-negative bacteria resistant to gentamicin and ampicillin appeared at the Hospital Clinico Universitario of Zaragoza. Most synthesized the novel class V aminoglycoside-(3)-*N*-acetyltransferase (8, 9). One of these strains (3644) contained pUZ3644, a plasmid of 160 kb that encoded Gm^r, Ap^r, Sm^r, sulfonamide resistance (Su^r), tetracycline resistance (Tc^r), and chloramphenicol resistance (Cm^r). This plasmid was transferable to *E. coli* J62 by conjugation. On prolonged storage, one of the transconjugants (J62/3644) lost the ability to transfer the resistances, although it still retained all of them, and plasmid DNA was no longer detectable in this strain. This result suggested that the plasmid or at least that part of the plasmid which was responsible for the resistances had been inserted into the chromosome.

Plasmid pUZ8 (Table 1) was introduced into J62/3644, and the resulting strain was then mated with *E. coli* J53. Gm^r transconjugants arose at a frequency of 10^{-8} and were all Ap^r. Analysis of one of these transconjugants showed that it contained a plasmid (pUZ999) of 79 kb that conferred Ap^r, Gm^r, kanamycin resistance (Km^r), and mercuric ion resistance (Hg^r). This result was consistent with insertion of a 27-kb segment of DNA that encoded Ap^r and Gm^r into the *tet* gene of pUZ8.

Plasmids pUZ999 and pACYC184 were then introduced into *E. coli* UB5201, and the resulting strain was mated with the *E. coli recA* strain AB2463. pACYC184 is not itself mobilized by pUZ8 (23); thus, any mobilization that does occur is likely to be due to transposition from the extra fragment in pUZ999, forming cointegrates. Cm^r transconjugants were obtained at a frequency of 2×10^{-7} and contained plasmids of 85 kb. These plasmids were stably maintained in *recA* strains, but in *recA*$^+$ strains, plasmids of 31 kb were segregated. These could be isolated by transformation to Gm^r with DNA from these *recA*$^+$ strains. One such plasmid (pUZ1000) was used for further study; it conferred Ap^r, Gm^r, and Cm^r.

Structure of pUZ1000. A map of pUZ1000 is shown in Fig. 1. The plasmid was a recombinant of pACYC184 with a 27-kb segment that encoded Gm^r and Ap^r; this segment was inserted into the *tet* gene of pACYC184. To locate the resistance genes, we constructed pUZ1001, pUZ1003, and pUZ1004. Plasmid pUZ1001 is a pUZ1000 deletion derivative obtained by recircularization of the 12.2-kb *Bgl*II fragment of pUZ1000. It contained the Ap^r and Gm^r genes from pUZ1000. The deletion did not affect the ends of the inserted DNA segment. pUZ1003 contained the 5- and 3.4-kb *Pst*I fragments of pUZ1000 and conferred Gm^r. Plasmid pUZ1004 was constructed by cloning the 2.9-kb *Sal*I fragment of pUZ1000 into the *Sal*I site of pACYC184 and conferred Gm^r and Ap^r.

The repetition of *Sal*I and *Pst*I sites at the termini of this segment (Fig. 1) suggested that it might be flanked by copies of the same sequence in direct orientation. To demonstrate the terminal repeat segments, we constructed plasmid pUZ1005, in which the 7.3- and 4.9-kb *Bam*HI fragments of pUZ1001 were joined in opposite orientation (Fig. 1), bringing the ends of the unit close together. When this molecule was denatured and self-annealed, a 0.8-kb stem-loop structure was evident (Fig. 2), confirming that repeat sequences of this length but directly oriented occurred in the original pUZ1000. We thought that this 0.8-kb sequence was probably an IS responsible for the transposition of Ap^r and Gm^r. That it was indeed an active IS was shown by analysis of recombinants formed between derivatives of pUZ1000 and pUZ8. For example, pUZ1002, which was constructed by recircularization of the 5-kb *Pst*I fragment of pUZ1000, is pACYC184 containing just one copy of the sequence (Fig. 1); this plasmid was mobilized by pUZ8, and the resulting recombinants were cointegrates in which pUZ8 and pACYC184 were fused by directly oriented copies of the sequence (data not shown). These results demonstrated that the 27-kb DNA insert present in pUZ1000 was a composite transposon. It will be called Tn*2922* in accordance with the recommendations of the Plasmid Reference Center (14).

Parts of the element have been sequenced to determine the origin of various sequences (Fig. 3). In region I, 176 base pairs (bp) of the 196 bp sequenced were identical to the end of IS*26* (17); the next 20 bp were the same as nucleotides 3297 to 3317 of the *tnpR* gene of Tn*3* (10). In region II, 90 bp were identical to nucleotides 3469 to 3559 of the *tnpR* gene of Tn*3* (10). In region III, the first 42 bp were identical to the terminus of Tn*3* (10); the other 14 bp were found in the 5′ untranslated region of the *aacC* gene of plasmid pWP14a (1). It seems, then, that the 27-kb segment of DNA present in pUZ1000 is flanked by copies of an element that is very similar to IS*26* and contains a fragment of Tn*3* that includes

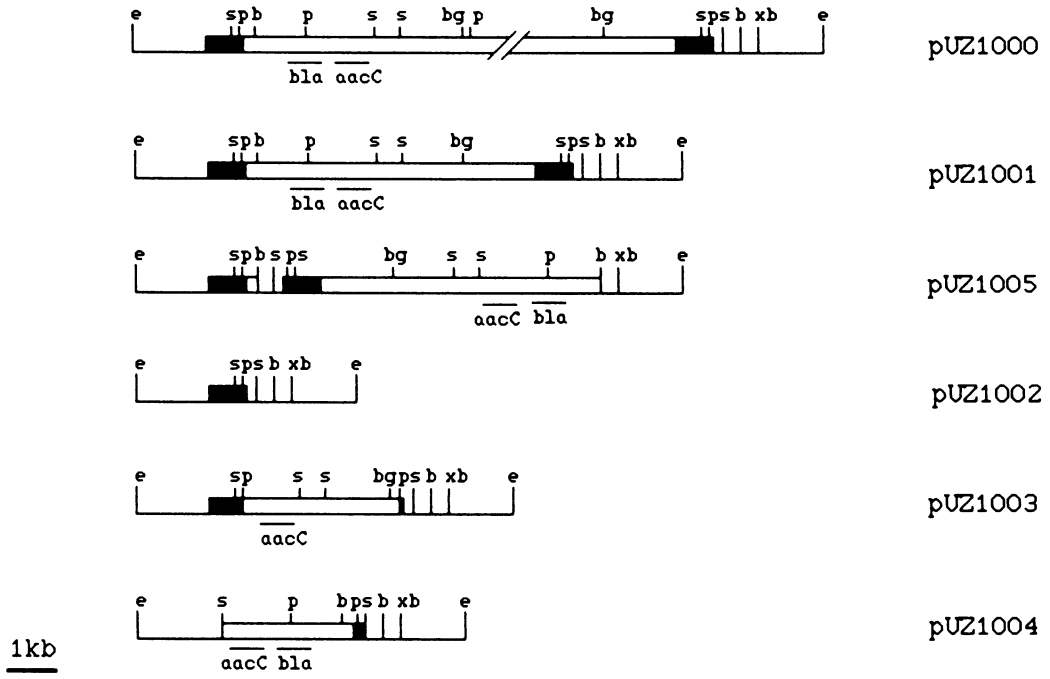

FIG. 1. Structures of plasmid pUZ1000 and its derivatives. The open boxes represent the 27-kb transposed segment, the solid boxes represent the 0.8-kb IS homologous to IS*26*, and the horizontal lines represent pACYC184. Abbreviations: b, *Bam*HI; bg, *Bgl*II; e, *Eco*RI; p, *Pst*I; s, *Sal*I; and xb, *Xba*I.

the *tnpR* and *bla* genes and, of course, the *aacC* gene. The arrangement of these sequences is shown in Fig. 3.

In a previously described Gm^r transposon (1), the *aacC* gene is transcribed from a promoter within IS*140*. This is unlikely to be the case here, since the fragment of Tn*3* intervenes between the gene and the relevant copy of IS*140* (Fig. 3). Transcription was investigated by examining the effect on the resistance pattern of transcriptional stops at various positions (Table 2). Terminations of transcription within the IS or the *tnpR* gene of Tn*3* had no effect on the

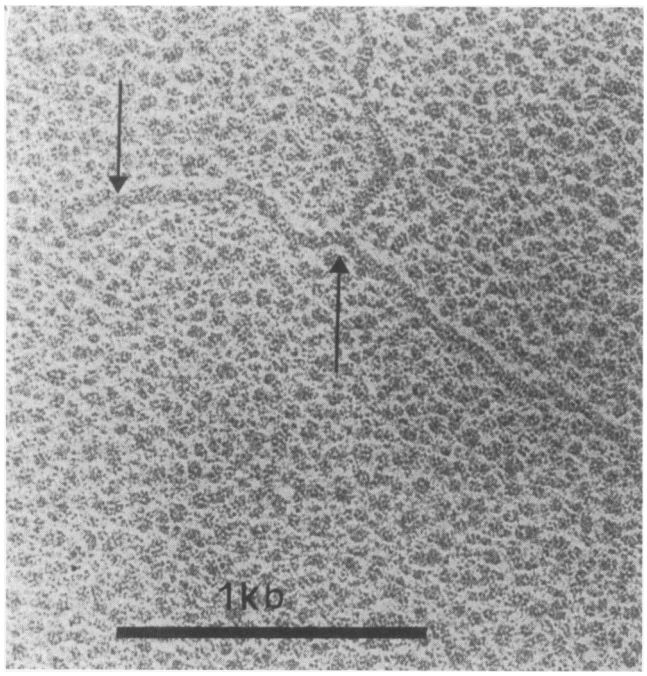

FIG. 2. Electron micrograph of a self-annealed, denatured pUZ1005 DNA molecule. The arrows indicate the double-stranded region. The bar represents 1 kb of double-stranded DNA.

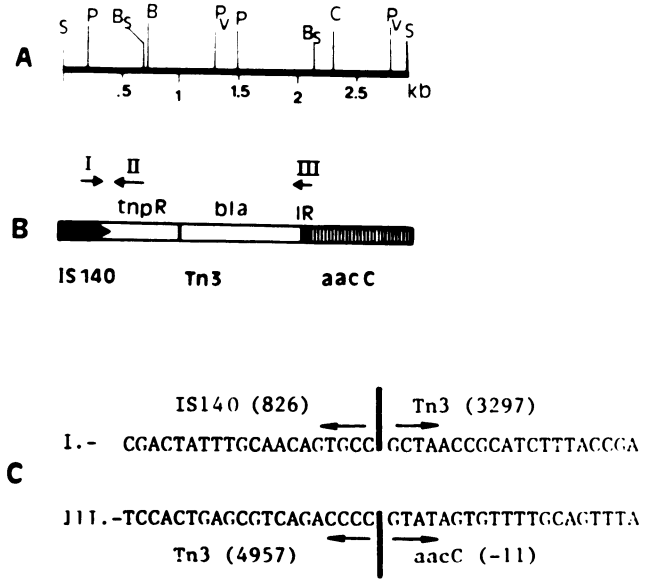

FIG. 3. (A) Restriction map of the 2.9-kb *Sal*I fragment of pUZ1000. Abbreviations: B, *Bam*HI; Bs, *Bst*EII; C, *Cla*I; P, *Pst*I; Pv, *Pvu*I; and S, *Sal*I. (B) Structural map of the fragment. The extent of the sequenced regions is indicated by arrows. I, II, and III refer to the regions discussed in the text IR, Inverted repeat. (C) Junction sequences between IS*140* and Tn*3* (I) and between Tn*3* and the *aacC5* gene (III) in pUZ1004. Only the sequences around the junctions are shown; the numbers in parentheses represent the base numbers at the junctions. Base pair numbers are as in reference 11 for IS*26*, reference 10 for Tn*3*, and reference 1 for the *aacC5* gene.

TABLE 2. Derivatives of pUZ1004 generated by Ω insertion mutagenesis

Mutant	Location of the insertion[a]	Phenotype
pUZ1004-1	30	Ap^r Gm^r
pUZ1004-2	435	Ap^r Gm^r
pUZ1004-3	813	Ap^r Gm^r
pUZ1004-4	1,400	Ap^s Gm^s
pUZ1004-5	2,100	Ap^r Gm^s
pUZ1004-6	2,511	Ap^r Gm^s

[a] Distance in base pairs from the *SalI* site of pUZ1004 (coordinate 0 in Fig. 3A).

resistance pattern; however, insertion within the *bla* gene resulted not only in Ap^s but also in Gm^s. This result strongly suggests that the *aacC* gene is transcribed from the *bla* promoter.

DISCUSSION

The 27-kb segment of DNA carrying determinants of Ap^r and Gm^r is flanked by directly oriented copies of an IS closely related to IS*26* (11). Thus, this segment could act as a composite transposon and has been called Tn*2922*. This arrangement of the IS element also indicates another way in which the complete segment can be inserted. This involves transposition of just one of the copies of the IS to yield a cointegrate; homologous recombination between appropriate copies of the IS would then generate a recombinant that contained just the 27-kb insert. The rearrangements described here probably occurred via this second route. For instance, when pUZ999 was used to mobilize pACYC184 (see Results), a molecule of 85 kb resulted. This molecule could have been composed of pUZ999 (79 kb), pACYC184 (4 kb), and an extra copy of the IS (0.8 kb), equalling 84 kb. The original insertion of pUZ3644 into the chromosome could easily be accomplished by cointegrate formation mediated by one of the copies of the IS.

Genetic rearrangements involving Gm^r and Ap^r genes have been found previously in plasmids pWP14a, pWP113a, and pWP116a isolated in the United States and Chile (3, 4). These plasmids contain IS*140*- and Tn*3*-like structures. In the case of plasmids pWP14a and pWP116a, it has been reported that the *aacC* genes use promoters from IS*140* (1, 4). The genetic organization found in pUZ3644 derivatives differs in that not all of the Tn*3* structure is present and also in the ability of a region containing Ap^r and Gm^r to be transcribed together using the promoter at the *bla* gene of Tn*3*.

The simultaneous occurrence in very separate geographic areas of a genetic organization involving an active IS (IS*140* or IS*140*-like), a region of Tn*3* including part of *tnpR*, the *bla* gene, and the right inverted repeat, and the genes encoding aminoglycoside-(3)-*N*-acetyltransferases is remarkable. The resulting composite may have independently evolved to produce an effective resistance determinant with two main properties, the ability to disseminate between replicons and the coordinate expression of resistance genes of two nonrelated drugs which are commonly used in the treatment of severe infections.

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