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Dissection of the r-determinant of the plasmid R100.1: the sequence at the extremities of Tn21

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ABSTRACT

We have sequenced the extremities of the transposon Tn21, isolated from the r-determinant of the multiple antibiotic resistance plasmid R100.1, and show that it is a member of the Tn3 family of elements.

INTRODUCTION

Multiple antibiotic resistance plasmids of the incFII class (R1drd19,R6 and R100.1) carry a region, known as the r-determinant (r-det), within which are located the majority of antibiotic resistance genes associated with the plasmid. The r-det is flanked by two directly repeated copies of the insertion element IS1 (1,2) and is itself composed of a series of intercalated transposable elements (see for example 3). On the basis of comparative studies of the r-det regions of these three plasmids and their derivatives, it seems likely that they have arisen by sequential acquisition of the various individual drug resistance determinants.

Kopecko and Cohen (4) have demonstrated that the r-det of pSC50, a derivative of R1drd19, carries two elements which transpose at relatively high frequencies. They are Tn3, the well characterised 4.9kilobase (kb) element which specifies resistance to ampicillin (Ap^r) (for a review see 5 and 6), and a larger, approximately 20kb, element which they called Tn4. The latter was shown to include Tn3 and to specify resistance to streptomycin (Sm^r) and sulphonamides (Su^r) as well as ampicillin. A transposon closely related to Tn4, called Tn21 (7), has been isolated

from the r-det region of the plasmid R100.1. This transposon specifies resistance to mercury salts (Hg^r) in addition to Sm^r and Su^r, but not Ap^r. Heteroduplex mapping and restriction analysis (7, 8, 9) show that Tn4 and Tn21 have a high degree of homology. They differ mostly by the presence of the Tn3 element within Tn4 inserted into the Hg^r determinant (10). Thus Tn4 has probably arisen from Tn21 by insertion of Tn3.

It has been suggested that Tn21 itself arose by the insertion of a region of DNA carrying Sm^r and Su^r genes into a smaller transposon carrying Hg^r (11). One candidate for such a progenitor of Tn21 is the element Tn501 (12). The transposon Tn501, together with γδ(Tn1000), IS101, Tn551, Tn1721 and Tn1771, are all closely related to Tn3 (see 5 and 6). They exhibit significant homology at the nucleotide sequence level, especially at their extremities. They all generate a direct repetition of five nucleotides in the target DNA on insertion and all generate unstable cointegrates between donor and recipient replicons as a step in the transposition process.

In order to determine whether Tn21 is related to the Tn3 family of elements and, more specifically, to determine whether it is derived from Tn501, we have determined the nucleotide sequence at its extremities and have compared these sequences to the known sequences of Tn501 and the other members of this class.

We find that Tn21 carries a 35/38 base pair (bp) homology in an inverted repeat configuration at its extremities. This sequence is closely related both to Tn3 and to Tn501. The sequence of Tn21 beyond the 38bp inverted repeats, however, diverges significantly from that found in either Tn3 or in Tn501. We conclude that Tn21 is a member of the Tn3 family of transposable elements but that it is as related to Tn3 as it is to Tn501. It is therefore unlikely that Tn21 is a direct result of insertion of Sm^r and Su^r genes into Tn501.

MATERIALS AND METHODS

Bacterial Strains.

The E. coli strains used in this study were LC799 (C_{600}

Nal^r, P_1^r, λ^r) and C600 containing pLC117 together with pBR322. The plasmid pLC117 is a Tc^s derivative of R100.1 (13).

Media.

Cells were grown in L broth (14) and plated on L agar. Where necessary, the medium was supplemented with nalidixic acid (Nal, 30μg/ml), chloramphenicol (Cm, 20μg/ml), streptomycin (Sm, 20μg/ml), tetracycline (Tc, 25μg/ml), ampicillin (Ap, 25μg/ml) and mercuric chloride (Hg, 20μg/ml).

Cointegrate Transfer.

Strain C600 carrying pLC117 and pBR322 was crossed with LC799 at 37^o overnight. Exconjugants were selected on L agar supplemented with Nal, Cm and Sm (to determine the frequency of transfer of pLC117) and Nal, Cm, Sm and Tc to determine the frequency of cointegrate transfer of pBR322 (see text).

Isolation of Plasmid DNA.

Plasmid DNA was isolated from cultures of LC799 exconjugants following amplification of the pBR322 derivatives with Chloramphenicol (150μg/ml) according to the method of Klein et al. (15).

DNA to be used in sequencing was prepared by fractionation of cleared lysates (16) on CsCl/EtBr gradients. In some cases the covalently closed circular plasmid DNA was subjected to a second purification in this manner.

Transformation.

DNA of pBR322 derivative plasmids isolated from the original LC799 exconjugants was reintroduced into C600 by transformation as described by Cohen et al. (17).

Restriction Enzyme Mapping and Gel Electrophoresis.

All restriction enzymes used in this study were purchased from New England Biolabs or Boehringer Mannheim. The conditions for digestion were as recommended by the manufacturers.

The conditions employed in both analytical and preparative acrylamide electrophoresis have been described previously (18), as have the methods used in the isolation of DNA fragments (18).

DNA Sequencing and Labelling Procedures.

The 5' ends of the purified RsaI fragments (fig 4) were

labelled, following phosphatase treatment, with [γ^{32}P]-ATP and T4 polynucleotide kinase as described in Hipskind and Clarkson (manuscript in preparation). The 3' ends were labeled using [α^{32}P]-dTTP and the Klenow fragment of DNA polymerase I (Boehringer Mannheim) (18).

The end labeled fragments were then sequenced according to the Maxam and Gilbert (19) chemical method. Both strands of the δH and δJ extremities of Tn21 in the insertion derivative pXZ1 were sequenced. The δJ extremity from the second insertion derivative pXZ5 was sequenced only from the 3' end as shown in figures 3 and 4.

RESULTS.

Isolation and Characterization of pBR322::Tn21 Insertion Derivatives.

In order to sequence the extremities of Tn21, we have chosen the fully sequenced cloning vector pBR322 (20) as a recipient plasmid. Insertions of Tn21 into this plasmid were isolated by the following method: strain C600 carrying both pBR322 and the conjugal plasmid pLC117, a Tc^s derivative of R100.1 (13), was crossed with strain LC799; Tc^r exconjugants, having presumably received pBR322 by means of a transposon-mediated replicon fusion with the conjugal plasmid, were selected. The transfer frequency of Cm^r Tc^r compared to that of Cm^r alone (pLC117) was determined to be approximately 5×10^{-6}. This is of the same order of magnitude as the frequency of pBR322 transfer promoted by the element γδ of the plasmid F under similar experimental conditions (21).

The cointegrate structure resulting from such a fusion is resolved into the two component plasmids, each carrying a copy of the transposon.

When plasmid DNA prepared from these strains was used to transform C600 to Tc^r, all of the resulting transformants tested proved to carry pBR322 with a Tn21 insertion. They were Hg^r and Sm^r but Cm^s showing that they contained only part of the pLC117 r-det (Fig. 1). Some were still Ap^r but some were Ap^s, presumably because of an insertion of Tn21 into the β-lactamase gene. Use of

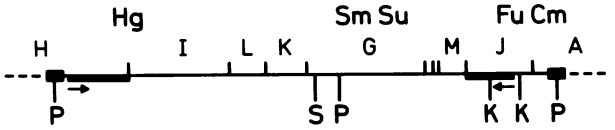

Figure 1. Restriction map of the r-det of R100.1.

The data are taken from references 22, 23, 25, 26 and 27. The map shows the restriction sites for KpnI(K), PstI(P), SalI(S) and EcoRI (unlabeled). The letters indicate the EcoRI fragments on the standard map of R100.1. The flanking copies of the IS1 elements are indicated by the small black boxes. The ends of Tn21 as determined in this study are indicated by heavy lines with the inverted repeats at the extremity represented by small arrows. The exact position of the end in fragments H (δH) and J(δJ) have not been determined.

Ap^r and Ap^s clones ensures that the derivative plasmids carry insertions in different locations in the pBR322 genome. EcoRI digests of two representative plasmids, obtained in this way, pXZ1 (Ap^r) and pXZ4 (Ap^s) are shown in figure 2 (lanes 2 and 5 respectively). Figure 2, lane 1 shows an EcoRI digest of the plasmid R100.1. We have previously shown that the EcoRI fragments G, I, J, K, L and M or R100.1 together with part of the IS1- carrying junction fragments A and H form the r-det region of the plasmid (22, 23) (fig. 1). Comparison with the derivatives pXZ1 and pXZ4 shows that the insertion spans a large fraction of the r-det. Both pXZ1 and pXZ4 carry fragments having the size of EcoRI fragments G(5.43kb), I(4.54kb), K(1.81kb), L(1.66kb) and M(1.28kb)of R100.1. In neither case is a fragment having the size of J observed. This indicates that one extremity of the transposon must be located within this fragment. Both plasmids have two additional fragments: 5.1kb and 4.2kb for pXZ1, and 5.8kb and 3.4kb for pXZ4. Since pBR322 has a unique EcoRI site, these fragments must each carry part of pBR322 and part of the transposon. The contribution of the transposon to the two pBR322 fragments must therefore be between 4.8 and 4.9kb. The overall length of the insertion, calculated by summation of the sizes of all fragments is thus approximately 19.6kb. This value has been confirmed by analysis of heteroduplex molecules formed between pBR322 and both insertion de-

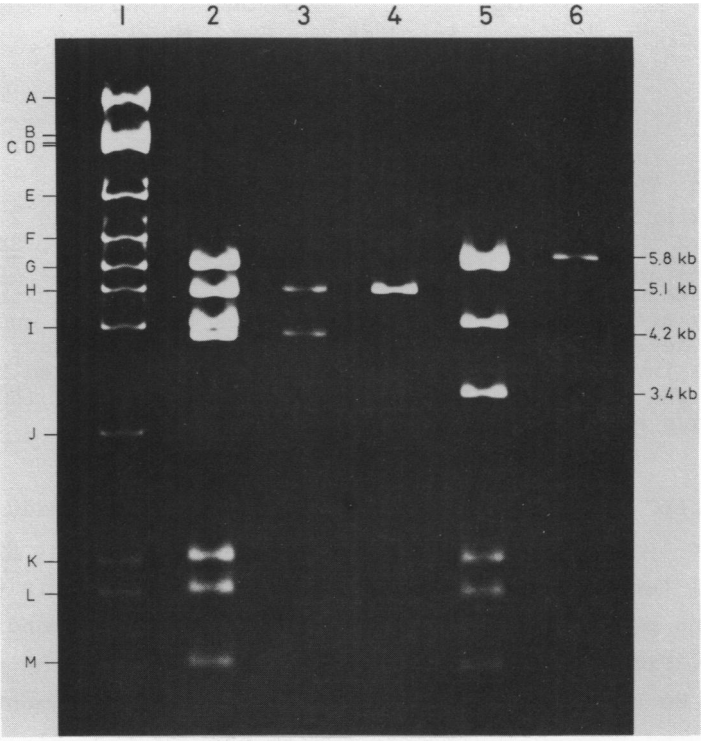

Figure 2. EcoRI digest of R100.1 and the pBR322::Tn21 insertion derivatives.

Lane 1 - 6: R100.1, pXZ1, pXZ2, pXZ3, pXZ4, pXZ5 respectively. The letters refer to the EcoRI fragments of R100.1 (25).

rivatives. Electron micrographs of such heteroduplexes demonstrated an insertion loop of 18.45 ± 1.35kb(n=30). The insertion loop also had a small (less than 100bp) stem structure (data not shown). Thus, the above analysis indicates that plasmids pXZ1 and pXZ4 carry an insertion which corresponds well with that expected for Tn21.

Fine Structure Mapping and in vitro Deletion of pXZ1 and pXZ4

In order to determine the orientation of Tn21 in both insertion derivatives, and to locate its point of insertion, we have subjected each plasmid to digestion with EcoRI and either KpnI, PstI or SalI. The r-det of R100.1 carries a single SalI

site, one PstI site and two KpnI sites (fig 1) while pBR322 carries single PstI and SalI sites and is not cleaved by KpnI (20). In the case of pXZ1, double digestion with EcoRI and each of these restriction enzymes indicated that the SalI site of pBR322 is located in the 4.2kb EcoRI fragment, while the unique PstI site is located in the 5.1kb fragment. Only a single KpnI site could be detected, indicating that one extremity of Tn21 is probably located between the two KpnI sites of the r-det (fig 1). This site occurs in the 4.2kb EcoRI fragment. The analysis (data not shown) defines the position and orientation of Tn21 in pXZ1 (Fig 3). An identical analysis was used to determine the struc-

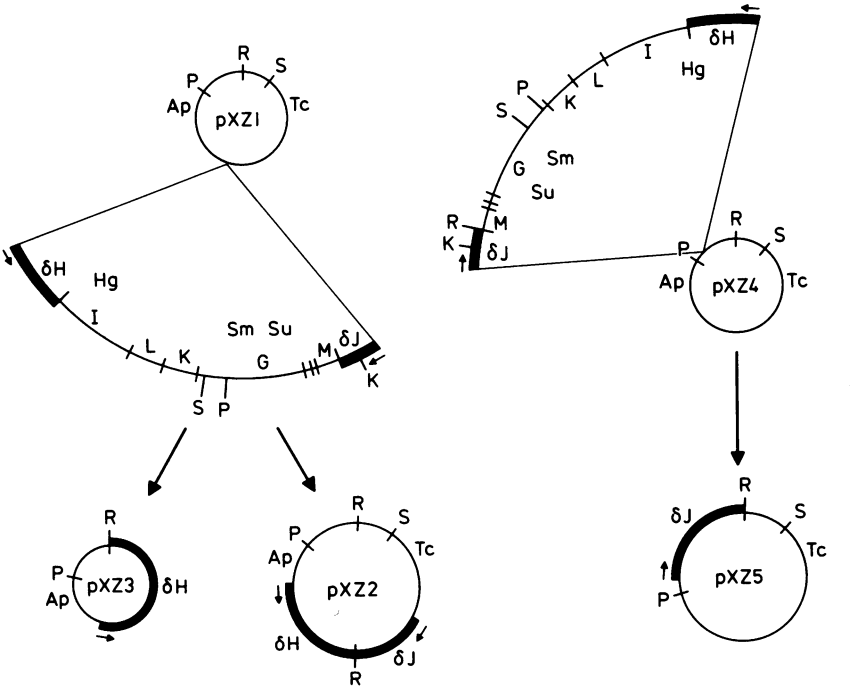

Figure 3. Structure of the pBR322::Tn21 derivatives.

The lengths of Tn21 and pBR322 are not drawn to scale. The restriction sites for KpnI(K), PstI(P) and SalI(S) are shown as are the EcoRI sites in Tn21 (unmarked) and the unique site in pBR322 (R). The ends of Tn21, marked δH and δJ, are derived from fragments H and J, respectively of R100.1. The inverted repeat sequences at their extremities are indicated by arrows.

ture of pXZ4 (Fig 3).

To facilitate the sequence analysis, we then reduced the size of both plasmids by digestion with EcoRI followed by religation. In the case of pXZ1, two plasmids, pXZ2 and pXZ3 were isolated following transformation of LC799. The plasmid pXZ3 specifies resistance to ampicillin alone while pXZ2 specifies both Ap^r and Tc^r. Digestion of these plasmids with EcoRI demonstrated that pXZ3 carries only the 5.1kb fragment of the parent plasmid and that pXZ2 carries both the 5.1 and 4.2kb pBR322::Tn21 junction fragments (figure 2, lanes 4 and 3). In the case of pXZ4, a single clone was retained for further study. This plasmid, pXZ5, carries only the 5.8kb pBR322::Tn21 junction fragment of the parent plasmid (Figure 2, lane 6).

A fine structure restriction map of these derivative plasmids was constructed, using standard techniques (Fig 4). Briefly, EcoRI/RsaI double digestions of the two insertion derivatives and the deleted plasmids were compared with that of pBR322. The results of these experiments allowed determination of the RsaI junction fragments in both the Ap^r (pXZ1) and Ap^s (pXZ4) inser--tion derivatives. In the case of pXZ1, these fragments were approximately 2120 and 550bp in length. The 550bp fragment was present in both pXZ2 and pXZ3, while the 2120bp fragment was present only in pXZ2. The results obtained with pXZ3 showed that the 550 bp fragment was delimited on one side by the RsaI site at co-ordinate 2283bp on the pBR322 map (20). This fragment was shown to carry a TaqI site within the Tn21 segment which was used in subsequent sequencing. Further restriction mapping of pXZ2 showed that both the PvuII site (pBR322 co-ordinate 2067) and an MspI site at 2154bp were present. This defines the Tn21 insertion into pXZ1 as having occurred between pBR322 co-ordinates 2154 and 2283.

The junction fragment between pBR322 and Tn21 in the Ap^s deletion derivative, pXZ5, was determined in the same analysis to have a size of 1650bp. It was shown to carry both the RsaI site at 2283bp and the unique PstI site at co-ordinate 3612bp on the pBR322 map. This defines the Tn21 insertion in the parent plasmid pXZ4, as having occurred between 3612 and 3932bp on the pBR322

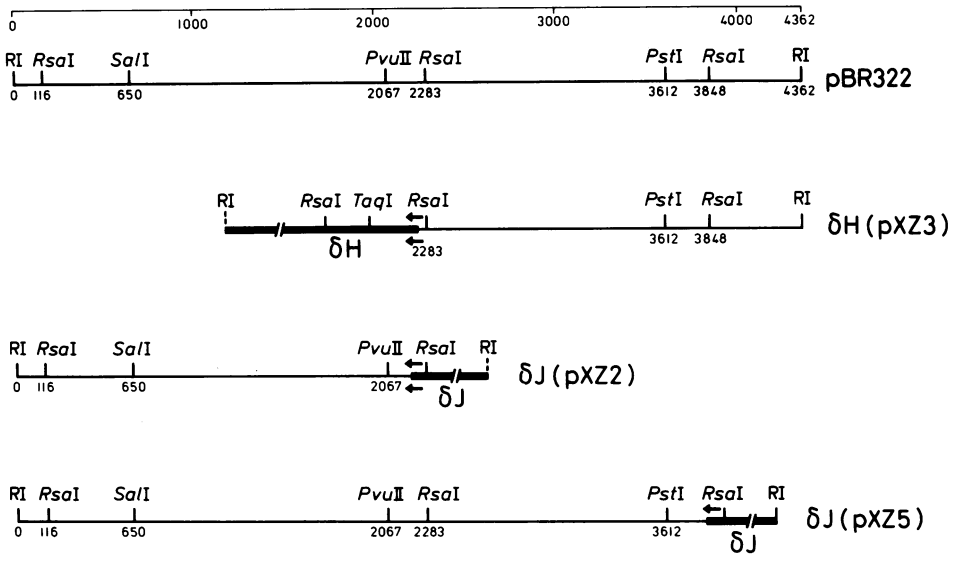

Figure 4. Fine structure restriction mapping and sequence strategy.

The structures of pXZ3, part of pXZ2, and pXZ5 are shown with relevant restriction sites. Heavy lines represent Tn21 and light lines pBR322 DNA. Only the RsaI sites within Tn21 closest to the junction with pBR322 is shown. Extremities δH and δJ from the Tn21 insertion in pXZ1 were isolated from pXZ3 and pXZ2 respectively. They were sequenced on both strands as indicated by the arrows. The δJ extremity from the Tn21 insertion in pXZ4 was isolated from pXZ5 and sequenced on one strand.

map.

Sequence Analysis of pXZ2, pXZ3 and pXZ5.

The strategy used to sequence the junction fragments between pBR322 and Tn21 is shown in figure 4. Both junction fragments of the insertion derivative pXZ1, isolated from pXZ2 (δJ in fig 3) and pXZ3 (δH in fig 3), were sequenced on both strands. The junction fragment δH from pXZ3 was sequenced from the RsaI site at pBR322 co-ordinate 2283bp (fig. 4) following end labeling and cleavage of the RsaI junction fragment with TaqI. The junction fragment δJ from pXZ2 was sequenced from the RsaI site internal to the transposon following end-labeling of the RsaI junction fragment and cleavage with PvuII. The junction fragment

δJ of pXZ5 was sequenced only on one strand from the RsaI site internal to the transposon (fig 4) following end-labeling and cleavage with PstI. The sequences obtained are shown in Figure 5.

DISCUSSION

The sequence data for the extremities of the 19.6kb transposon Tn21 (fig 5) indicate that this transposon shares many features in common with members of the Tn3 family of elements. Tn21 carries a 35/38bp homologous inverted repeat at its extremities (fig 6) which exhibits strong but incomplete homology with those of Tn3 and Tn501 (fig 7). Comparison of the extremities of all elements of the Tn3 family sequenced to date reveals certain highly conserved regions (5,6). Each extremity is delimited on its 5' end by four (or in the case of Tn501, five on one side and six on the other) G residues. At least one, and in most cases both, extremities contain a sequence 5'-T.A.A.G-3' located between 32 and 35bp from the 5' end at the 3' extremity of the inverted repeat. Each element also carries a six base pair sequence:

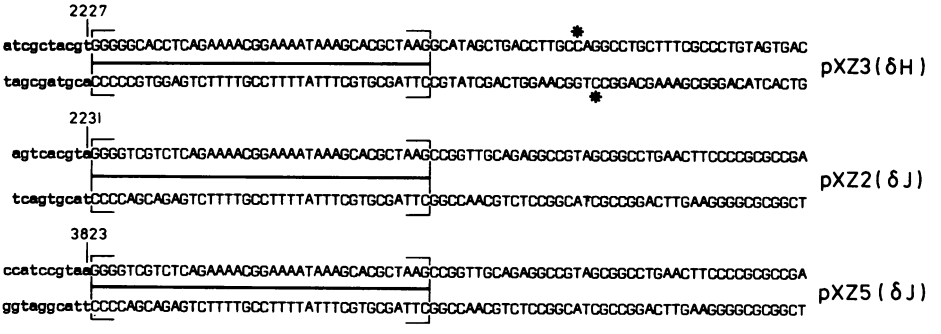

Figure 5. Sequence of the extremities of Tn21.

Lower case letters represent pBR322 DNA. The pBR322 coordinates (2O) at the point of insertion are indicated. The 38bp inverted repeat region at the extremities is also shown. We have assumed, in assigning the ends, that Tn21 produces a 5bp repeat in the target DNA on insertion. One ambiguity arose due to the presence of methylated cytosine residues in the δH junction at positions 55 (5'-3') and 57 (3'-5'). These form part of an EcoRII site and were clearly visible from the sequence data of both strands. The methylated-C residues are indicated by an asterix.

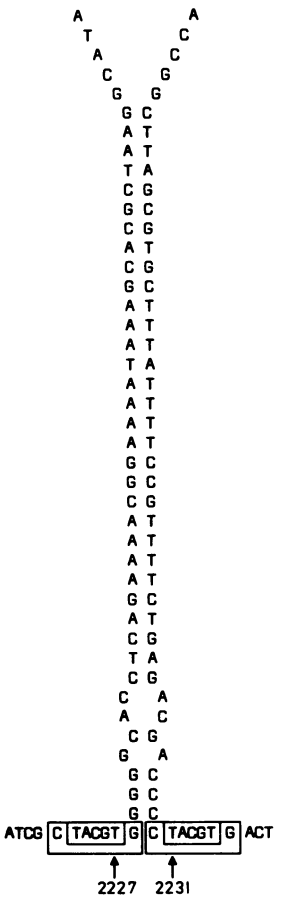

Figure 6. The inverted repeat sequences of Tn21.

This figure shows the homology between both extremities of Tn21 in plasmids pXZ2 and pXZ3. The point of insertion into pBR322 is indicated and assumes that Tn21 generates a 5bp repeat in the target DNA on insertion (shown within the smaller boxes). The larger boxes are included to show the possible seven base pair repetition (see text).

5'-C.G.Pu.Pu.A.A-3' located between 14 and 21bp from the 5'end. The extremities of Tn21 show all these features (fig 7) Tn21, however, diverges from Tn501 as widely as it does from Tn3. Comparison of the sequence of Tn21 and Tn501 in the 3' direction from the end of the repeated segment indicates an even wider divergence (fig 7). This implies that Tn21 cannot be derived directly from Tn501. In this light, it is interesting to note that the elements Tn1721 and Tn1771 show complete homology with the 38bp inverted repeats of Tn501 and that this homology extends a significant distance 3' to the repeats (24). Tn21 is thus less related to Tn501 than are either of these two elements.

All members of the Tn3 family of elements analysed thus far

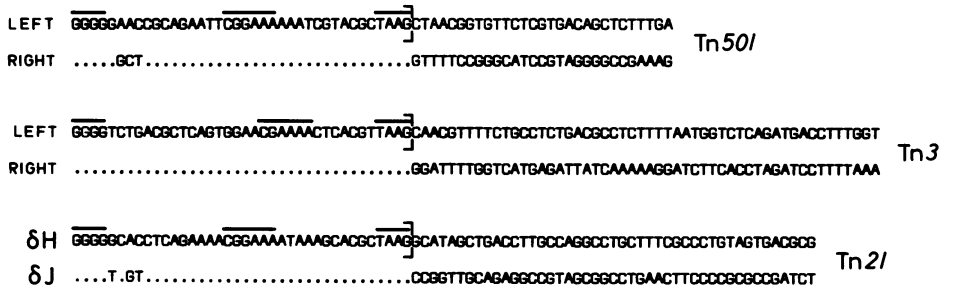

Figure 7. Comparison of Tn3, Tn21 and Tn501.
The sequence of the extremities of Tn3 (28), Tn501 (24) and Tn21 are shown. Only the non homologies in the second end of each element are indicated. The three sequences in each element marked are common to all elements in the Tn3 family.

have been found generate 5bp direct repeats in the target DNA on insertion (5,6). The data we have obtained from the sequences of both extremities of Tn21 in pXZ1 and one extremity in pXZ4 are fully compatible with a five base pair repeat. However we cannot rule out the possibility of a 6 or 7bp repeat (fig 6). It seems unlikely that Tn21 generates a 7bp repeat on insertion since this would define one end of the element as a sequence of three C residues. All other members of the Tn3 family carry four (or in the case of Tn501, five and six) C residues at their 3' ends. Based on the close sequence relationship between members of the Tn3 family and Tn21 it seems most likely that Tn21 generates a 5bp repeat.

The direct repeat found in both the insertion derivatives contains a sequence 5'-T.A.C.G-3'. This sequence occurs 18 times in pBR322. The probability of an insertion occuring next to this sequence in two independent events is low and may suggest some specificity in the choice of target site. Further studies will be required to determine if this is the case.

In summary, Tn21 is a member of the Tn3 family of transposable elements but it was not derived directly from Tn501.

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