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Characterization of IS*46*, an Insertion Sequence Found on Two IncN Plasmids

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The IncN plasmids R46 and N3 each contain two copies of an insertion sequence which we denote IS*46*. This insertion sequence has single *Pst*I and *Sal*I restriction sites and is 0.81 kilobases long. All four copies of IS*46* were capable of forming cointegrates, although the DNA between the insertion sequences, which in each case carries a tetracycline resistance gene, was not transposable in the form of a compound transposon. IS*46*-mediated cointegrates resolved in Rec$^+$ but not in RecA$^-$ cells. Recombination between two copies of IS*46*, causing an inversion, accounts for the existence of two distinct forms of R46. IS*46*-mediated deletions were probably responsible for the formation of the plasmid pKM101 from R46. IS*46* was not homologous to IS*1* but did show homology with IS*15*.

Insertion sequences form one important group of transposable DNA elements; they differ from transposons in carrying no readily detectable marker and in being usually smaller in size (for a review, see ref. 34). Insertion sequences have been found on the chromosomes of *Escherichia coli* and other enterobacteria and in plasmids either as individual elements or in pairs bounding antibiotic resistance markers, forming composite transposons.

Only a relatively small number of plasmids of various incompatibility groups have so far been screened for the presence of insertion sequences. In the course of our studies on the IncN plasmids R46 and N3 (8; A. Brown, Ph.D. thesis, University of Edinburgh, Edinburgh, Scotland, 1981), we encountered phenomena suggesting that each plasmid carries two copies of an insertion sequence. In both R46 and N3 these insertion sequences bound the Tc^r region of the plasmid in inverted and direct repeat orientations, respectively. This paper describes these experiments and others designed to characterize the sequences (called IS*46*) to show that they are interrelated and to determine their relationship to insertion sequences described previously. Also, unsuccessful attempts were made to transpose the Tc^r determinants of R46 and N3 as composite transposons.

MATERIALS AND METHODS

Bacterial strains, plasmids, and phages. The characteristics of *E. coli* K-12, *Enterobacteriaceae*, and *Pseudomonas* strains, and of their plasmids and bacteriophages, are described in Table 1.

Media and buffers. Culture media were described by Willetts and Finnegan (67), and antibiotic additions were described by Brown and Willetts (8).

Genetic techniques. Quantitative membrane filter matings were carried out as described by Brown and Willetts (8), and transformation was carried out by the technique of Lederberg and Cohen (41). P1 *vir* a transduction was by the method of Willetts et al. (66).

DNA preparation and manipulation. Plasmid DNA was prepared by a procedure based upon the cleared lysate technique of Clewell and Helinski (12) and the polyethylene glycol precipitation technique of Humphreys et al. (30). It was then purified by cesium chloride-ethidium bromide density gradient centrifugation. Small-scale plasmid preparations were made by the method of Birnboim and Doly (5).

Bacterial DNA was prepared as described by Willetts et al. (66). Large-scale preparation of lambda phage lysates and extraction of the phage DNA were described by Willetts (63).

The use of restriction enzymes for cleavage analysis and cloning was described by Brown and Willetts (8).

Southern hybridization techniques. For hybridizations, DNA fragments separated on agarose gels were denatured and transferred to nitrocellulose filters by the methods of Southern (57). The filters were rinsed in 2× SSC (1× SSC is 0.15 NaCl plus 0.015 M sodium citrate) and baked at 80°C for 2 h. Plasmid DNA was nick translated as described by Willetts et al. (66), after Rigby et al. (52). The 195-base-pair (bp) *Sal*I-*Pst*I fragment of IS*46* was also labeled by this method after extraction from an 8% acrylamide gel by the method of Maxam and Gilbert (43).

The nitrocellulose filters were presoaked for 1 h by shaking at 37°C in hybridization fluid: 4× SSC (0.6 M NaCl, 0.06 M sodium citrate), 50% formamide, 0.1% sodium dodecyl sulfate, and 1× Denhardt solution (0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, 0.02% Ficoll). Hybridization was then carried out overnight at 37°C in a plastic bag containing hybridization fluid (20 ml for a 12-lane gel) plus 500 μg of denatured salmon sperm DNA and 10^5 to 10^6 cpm of denatured probed.

After the hybridization, filters were washed twice for 1 h under hybridization conditions and twice for 1 h in 2× SSC. The filters were then dried at 37°C. Autoradiograms were usually exposed for 1 to 2 days at −70°C, using preflashed X-ray film (Dupont Cronex 4) and a phosphotungstate intensifying screen (Dupont Cronex).

Electron microscopy. Self-annealing, heteroduplexing, and formamide spreading of DNA molecules were by the method of Davis et al. (18). The molecules were spread on Parlodion grids, and φX174 double-stranded (5.38 kilobases [kb]) and M13 single-stranded (6.23 kb) DNA circles were added to provide length standards. Grids were examined, and molecules were photographed with a Siemens Elmiskop 101

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TABLE 1. Bacterial strains, plasmids, and phages

Sample	Relevant characteristics	Reference
Bacterial strains		
Escherichia coli K-12 C600	Leu$^-$ Thi$^-$ Thr$^-$	2
Escherichia coli K-12 ED24	Spc^r	67
Escherichia coli K-12 ED395		19
Escherichia coli K-12 ED3818	His$^-$ Lys$^-$ Trp$^-$ Str^r Nal^r	68
Escherichia coli K-12 ED3886	RecA$^-$ Spc^r	*recA56* derivative of ED24
Escherichia coli K-12 ED8654	Met$^-$	6
Escherichia coli K-12 JC3272	His$^-$ Lys$^-$ Trp$^-$ Str^r	1
Escherichia coli K-12 JC6310	His$^-$ Lys$^-$ Trp$^-$ Str^r RecA$^-$	64
Escherichia coli B		59
Enterobacter aerogenes (ATCC 13048)		54
Proteus morganii (ATCC 25830)		54
Providencia stuartii 164		55
Salmonella typhimurium LT2 (NCIB 10248)		13
Shigella sonnei		J. Govan (personal communication)
Pseudomonas aeruginosa PAO2	Ser$^-$	28
Plasmids		
pBR322Δ::IS*1*	pBR322 deletion plasmid carrying IS*1*	M. Chandler (personal communication)
pCU1	IncN Ap^r Spc^r	35
pED899	Sul^s Tra$^-$ R46	This paper
pED904	in vitro deletion of R46 containing *Hin*dIII A fragment	8
pED1012	pBR322 derivatives containing N3 *Bgl*II E fragment in the *Bam*HI site	This paper
pED1013	As for pED1012, but with N3 *Bgl*II E fragment in opposite orientation	This paper
pED1017	Tra$^-$ N3	This paper
pED1022	pME420::IS*46*(d)	This paper
pED1030	R46::pME420 cointegrate	This paper
F		27
pIP1091	pBR322 derivative carrying IS*15*Δ	36
pME420	pBR325 derivative with a 1.0-kb deletion of the Tc^r gene removing the *Hin*dIII, *Bam*HI, and *Sal*I sites	J. Watson (personal communication)
pML31	*Eco*RI f5 fragment of F linked to the *Eco*RI fragment of R6-5 carrying Km^r	60
N3	IncN Sul^r Spc^r Tc^r *Eco*RII Uvp$^+$	61
R1-19	IncFII Ap^r Cm^r Km^r Sm^r Sul^r	46
R46	IncN Ap^r Sul^r Spc^r Tc^r Uvp$^+$	16
R68	IncP Ap^r Km^r Tc^r	10
R100	IncFII Cm^r Hg^r Sm^r SuT^r Tc^r	21
R388	IncW Sul^r Tp^r	17
R390	IncN Ap^r Spc^r Sul^r Tc^r *Eco*RII	14
RSF1010	IncQ Sm^r Sul^r	24
Phages		
EDλ4	λ *b515 b519 c*I857 *Sam7*	19
VAλ3	EDλ4 *exo*::IS*1*	66
P1 *vir* a	Virulent variant of P1 *kc*	65

electron microscope. Molecules were measured with a Ferranti Cetec digitizer with an Olivetti P6040 minicomputer.

RESULTS

R46 contains short regions of homology. Linear single-stranded molecules of R46 were obtained by cleaving R46 DNA at its unique *Xho*I site and denaturing at high pH. After a brief period of reannealing, the molecules were spread and photographed in the electron microscope. The structures observed (e.g., Fig. 1) showed that R46 contains two pairs of inverted repeat sequences. One pair formed a short double-stranded stem of ca. 340 bp with a single-stranded loop of ca. 7.3 kb. The dimension and location of this stem-loop structure indicate that it is equivalent to that previously observed by Langer et al. (39) on pKM101, a deletion mutant of R46 (47). This pair of short inverted repeats flanks the *mucAB* genes (51) and possibly the replication region of the two plasmids (Fig. 2A).

The second pair of inverted repeats formed a double-stranded region of 0.86 ± 0.03 kb and were separated by ca. 9.4 kb of DNA which forms the two single-stranded "tails" in Fig. 1. One of these tails is very short, indicating that one copy of the repeat lies very close to the *Xho*I site in the region of R46 that is deleted in pKM101 (Fig. 2A; 8). This second pair of inverted repeats would not, therefore, have been detected by Langer et al. (39). The position of each copy of this repeat on R46 coincided with closely spaced cleavage sites for *Sal*I and *Pst*I, suggesting that these sites are contained within the repeated sequence.

Inversion of a region of R46 flanked by inverted repeats. The sizes of the restriction endonuclease cleavage fragments of R46 DNA have been measured independently by Brown and Willetts (8) and by Langer and Walker (40), and there were striking differences between some of the values obtained by the two groups. The existence of the second pair of inverted repeats described above could account for these discrepancies, since recombination between the two copies of this sequence would invert the intervening 9.5-kb segment, producing two distinct forms of R46. We designated our form "R46A" and designated that of Langer and Walker "R46B" and confirmed the differences in restriction fragment sizes by direct comparison on agarose gels (Fig. 3). R46A and R46B had similar total sizes and contained the same total number of cleavage sites for each restriction enzyme used. For *Kpn*I and *Sma*I, for which there are no

sites within the 9.5-kb segment, the digest patterns were identical, whereas for others (*Bam*HI, *Bgl*II, *Eco*RI, and *Hin*dIII) most fragments were common, but two fragments in each case differed between R46A and R46B. For example, the 17.1- and 8.5-kb *Bgl*II A and B fragments of R46A were replaced by fragments of 22.0 and 3.6 kb in R46B. Analysis of the sizes and the map positions of such altered fragments confirmed that the two forms of R46 differ by inversion of the 9.5-kb segment. This inversion most probably results from reciprocal recombination between the homologous inverted repeats. Although the inverted segment contains sites for *Sal*I and *Pst*I, the restriction digest patterns for these two enzymes were indistinguishable between R46A and R46B. This further supports the inference that the repeated sequence itself contains a site for each of these enzymes (Fig. 2A).

Evidence for similar short regions of homology on N3. A restriction endonuclease cleavage map of the IncN plasmid N3 was constructed by techniques similar to those used for R46 (8). A combined physical and genetic map of N3 is presented in Fig. 2B; a description of the data from which it was derived will be presented elsewhere (G. Coupland, A. Brown, and N. Willetts, manuscript in preparation).

The map shows that there are two pairs of *Sal*I and *Pst*I sites (characteristic of the inverted repeat sequences found in R46) in N3, though repeated in direct, rather than inverted, orientation. Both small *Sal*I-*Pst*I fragments from N3 were shown to comigrate on acrylamide gels with the *Sal*I-*Pst*I fragment originating from near coordinate 36 kb in R46. This suggested that N3 carries two copies of a repeated sequence similar to those found on R46. Further evidence for this resulted from consideration of heteroduplex molecules formed between single strands of N3 and R46; in these molecules a short (0.7 ± 0.05 kb) region of heteroduplex DNA formation was apparent which corresponds in position to a part of one of the R46 repeat sequences, and to a complementary region on N3 located between coordinates 51 to 52 kb, that includes both *Sal*I and *Pst*I sites (Fig. 2B; Coupland et al., in preparation).

During the course of our studies of N3, a spontaneous Tc^s deletion mutant (pED991) was detected fortuitously. *Bgl*II digests showed that ca. 11 kb of DNA had been lost, and *Pst*I digests showed that the endpoints of the deletion lie close to the *Pst*I sites at coordinates 51.75 and 5.4 kb (Fig. 2B). No novel-sized *Pst*I or *Sal*I fragments were present, suggesting that pED991 arose by homologous recombination between the directly repeated sequences covering these coordinates, leading to excision of the intervening DNA.

The four repeated sequences on R46 and N3 are shown below to have the transposition properties of an insertion sequence. We shall therefore refer to them as IS*46*(a) and IS*46*(b) (mapping near coordinates 36 and 46 kb on R46) and IS*46*(c) and IS*46*(d) (near coordinates 51 and 5 kb on N3), respectively.

IS*46* will fuse R46 or N3 with a second plasmid to form cointegrate molecules. One consequence of the presence of an insertion sequence in a plasmid is that it will fuse this plasmid with another replicon to form a cointegrate molecule with directly repeated copies of the insertion sequence present at each interreplicon boundary (25, 50). Such cointegrates are often resolved into their component plasmids at only low frequencies, even in Rec^+ hosts (4, 23). Consequently, conjugative plasmids carrying insertion sequences can mobilize nonconjugative plasmids as part of cointegrate molecules. We used this system to determine whether the four repeated sequences decribed in the previous section were transposable. An insertion sequence may also transpose directly, but this is very difficult to measure and was not attempted in the present case.

A *recA* host strain (JC6310) carrying R46 or N3, and the monomeric form of the nonconjugative plasmid pME420 (a Cm^r Tc^s deletion derivative of pBR325), was used as the donor in matings with the Rec^+ Nal^r recipient strain ED3818. The frequencies of formation of Tc^r [Nal^r] and Cm^r [Nal^r] transconjugants were taken as measures of R46 or N3 and pME420 transfer, respectively.

The strain containing R46 and pME420 gave Cm^r transconjugants at a frequency of 3×10^{-5} relative to R46

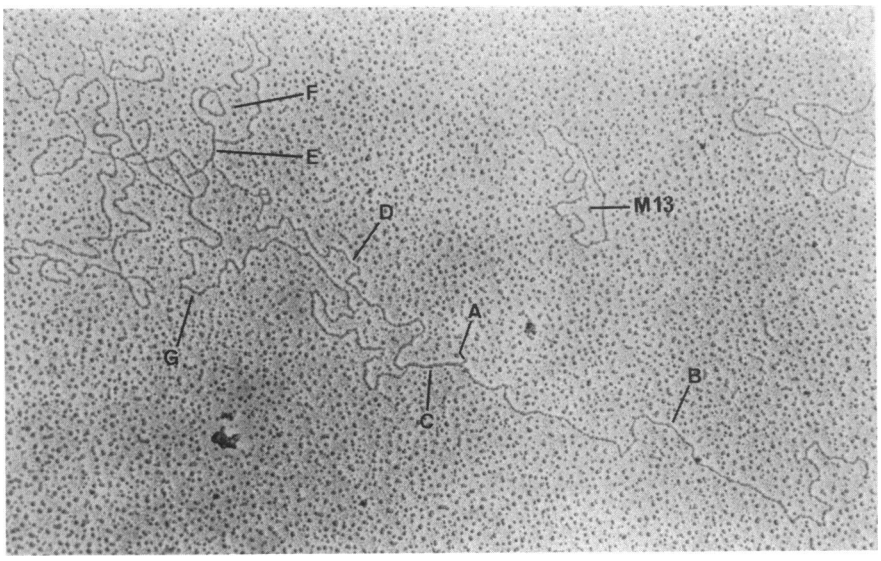

FIG. 1. Self-annealed R46 single-strand DNA after cleavage of the plasmid with *Xho*I. Two double-stranded "snap-back" regions are visible, of lengths 0.86 and 0.34 kb, labeled C and E, respectively. The lengths in kilobases of the single-stranded segments of the molecule are as follows: A, 0.06; B, 9.4; D, 7.6; F, 7.3; and G, 23.8. A single-stranded M13 size standard is marked.

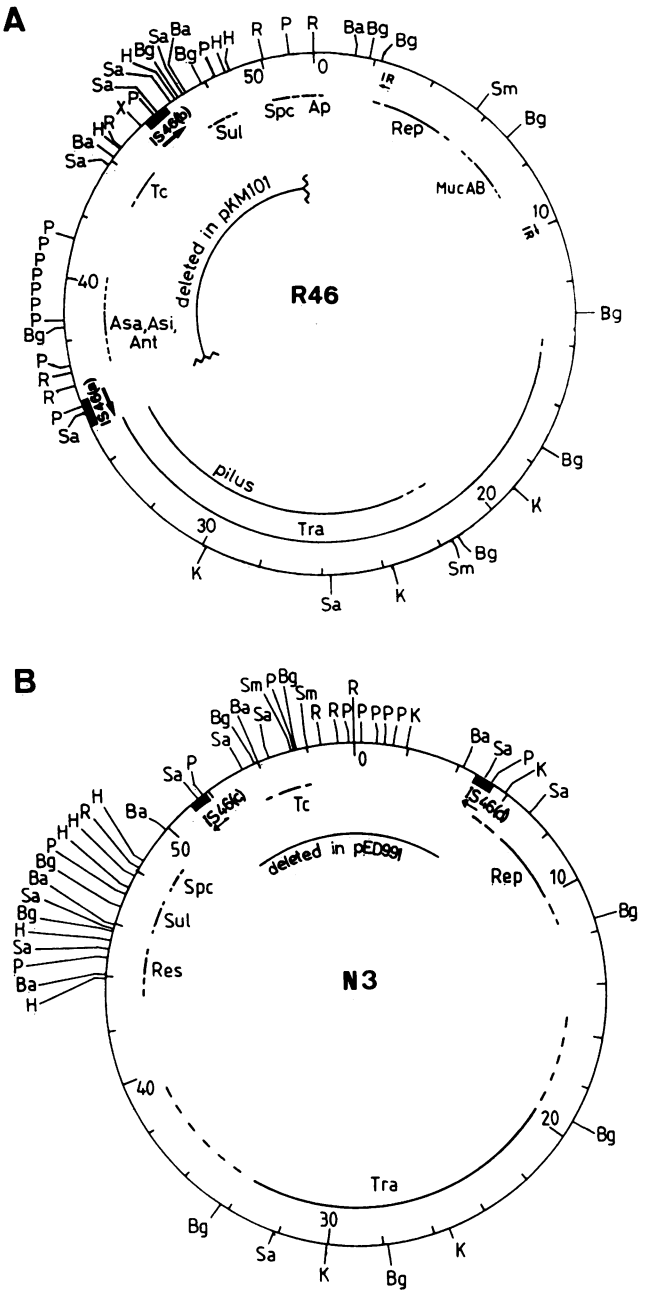

FIG. 2. (A) A map of R46. Redrawn from Brown and Willetts (8) with the addition of the two copies of IS*46* (drawn with heavy lines), and of the 0.34-kb inverted repeats (I.R.). Abbreviations are as follows: Ap, ampicillin; Asa, arsenate; Asi, arsenite; Ant, antimony; MucAB, enhanced mutagenesis; Rep, replication; Spc, spectinomycin; Sul, sulphonamide; Tc, tetracycline; and Tra, conjugal transfer. As well as the previously reported arsenate resistance, R46 confers resistance to arsenite ions and antimony III. The restriction enzyme cleavage sites shown are *Eco*RI (R), *Hin*dIII (H), *Bgl*II (Bg), *Pst*I(P), *Sal*I(S), *Bam*HI (Ba), *Kpn*I (K), *Sma*I (Sm), and *Xho*I (X). Coordinates are marked in kilobases. Map coordinates of restriction fragments referred to in the text are as follows: *Bgl*II-A, 21.3 to 38.4; *Bgl*II-B, 38.4 to 46.9; and *Pst*I-F, 36.0 to 37.2. (B) A map of N3. A detailed description of the data that allowed construction of this map will be presented elsewhere (Coupland et al., in preparation). The phenotype of N3 differs from that of R46 in that it does not carry Ap^r, Asa^r, Asi^r, or Ant^r determinants, its Tc^r gene is different (8, 45), and it encodes the *Hsp*II restriction and modification system. The copies of IS*46* are marked with heavy lines. Abbreviations are as for Fig. 2A; Res, restriction modification. Map coordinates of fragments mentioned in the text are as follows: *Bgl*II-A, 55.4 to 11.7; and *Bgl*II-E, 46.6 to 53.9.

transfer: all of these showed the antibiotic resistance phenotypes of both plasmids. Small-scale plasmid DNA preparations were made from 15 independent transconjugants and were digested with *Bgl*II. Cointegrates resulting from transposition of IS*46*(a) and IS*46*(b) were expected to have pME420 integrated within the *Bgl*II B and *Bgl*II A fragments of R46, respectively. In nine of the isolates, *Bgl*II-B had been replaced by a larger fragment (pME420 contains no site for *Bgl*II), whereas in the other six it was *Bgl*II-A that was replaced. This suggested that IS*46*(a) and IS*46*(b) could each promote cointegrate formation (i.e., transpose) at approximately similar frequencies. *Sal*I digests showed that the cointegrate plasmids contained the normal complement of R46 *Sal*I fragments, plus an extra 5.3-kb fragment arising from pME420 (which has no *Sal*I site) flanked by directly repeated copies of IS*46*. One of the IS*46* elements has therefore been duplicated in each case. These data confirmed that the Cm^r transconjugants contained R46::pME420 cointegrates formed by transposition of IS*46*. Restriction digests of two representative cointegrates formed via IS*46*(a) and IS*46*(b) are shown in Fig. 4A.

In analogous experiments, pED904 was substituted for R46. pED904 is an in vitro deletion mutant of R46 (8), which carries IS*46*(a) but not IS*46*(b). This plasmid mobilized pME420 at a frequency of 8×10^{-6} relative to its own transfer, and *Bgl*II digests of plasmid DNA from Cm^r transconjugants showed them to contain pED904::pME420 cointegrates formed via IS*46*(a). IS*46*(a) is therefore capable of cointegrate formation (i.e., transposition) in the absence of IS*46*(b). A derivative of R46 carrying IS*46*(b) alone was not available to allow the independent transposition of this copy to be tested.

N3 also mobilized pME420, at a frequency of 1.5×10^{-5} of its own transfer. Plasmid DNA from 12 independent Cm^r transconjugants was examined by digestion with *Bgl*II and *Sal*I, and all 12 carried cointegrates of N3 and pME420 formed by transposition of IS*46*(d) (Fig. 4A). This copy of IS*46*, therefore, transposed more frequently than IS*46*(c) in these experiments, although results in the following section show that IS*46*(c) is also capable of transposition.

Cointegrate formation between R388 and *tra*$^-$ derivatives of R46 or N3. Although the mobilization technique used above provides an effective test for the presence of insertion sequences on conjugative plasmids, it would not detect direct transposition of antibiotic resistance markers by a mechanism independent of cointegrate formation. Since the tetracycline resistance markers in both R46 and N3 are flanked by copies of IS*46* (Fig. 2A), and these combinations might form composite transposons, we wished to look for direct transposition of these. The conjugation protocol was therefore reversed by using R388 (a conjugative IncW Tp^r Sul^r plasmid with no known transposable sequences) to mobilize the Tc^r markers of transfer-deficient mutants of R46 and N3.

A JC6310 derivative carrying R388 and pED889 (a *tra*$^-$ point mutant of R46 with a residual transfer frequency of $<10^{-7}$) transferred the pED889 Tc^r marker to ED3818 at a frequency of 10^{-6} compared with R388 transfer. Each transconjugant carried all of the pED889 antibiotic resistance markers plus Tp^r, suggesting that they carried cointegrates

of the two plasmids. This was confirmed by *Bgl*II digestion of plasmid DNA from six representative transconjugants, and the fragment pattern further showed that one cointegrate has arisen via IS*46*(a) transposition, and five have arisen via IS*46*(b) transposition. Digests of two cointegrates are shown in Fig. 4B. A large number of the Tc^r transconjugants from the above mating were then screened to search for occasional transposition of the segment flanked by IS*46*(a) and IS*46*(b) as a Tc^r and As^r transposon. Of 350 Tc^r colonies tested, 3 were Tp^r Ap^s Spc^s, the pattern expected to result from direct transposition of such a transposon. However *Bgl*II restriction analysis showed that all three plasmids carried much larger segments of R46 DNA and were deletion derivatives of cointegrate molecules. These data, plus the observation that none of the 21 cointegrates examined contained duplications of the entire IS*46*(a)-IS*46*(b) region, indicate that transposition of this region occurs (if at all) considerably less frequently ($<10^{-9}$ per R388 transconjugant) than that of IS*46* itself.

Similar experiments were carried out for N3, using pED1017, a *tra*$^-$ N3 mutant deficient in pilus formation with a residual transfer frequency of 5×10^{-7}. A JC6310 derivative carrying R388 and pED1017 transferred the Tc^r marker of the latter plasmid at a frequency of 9×10^{-6}, compared with R388 transfer. All of the transconjugants carried all of the pED1017 antibiotic resistance markers as well as Tp^r, but in most, pED1017 and R388 were present as separate plasmids. This may to be due to a low level of complementation of the transfer defect of pED1017 by R388. Clones carrying cointegrate plasmids were identified amongst the transconjugants as those which could retransfer Tc^r at high frequency in replica matings with ED24. They constituted ca. 6% of the initial Spc^r Tc^r Tp^r transconjugants. DNA from 11 of these was digested with *Bgl*II to confirm that they were cointegrates: of these, 8 were formed via IS*46*(c), and three were formed via IS*46*(d) (Fig. 4B). In these matings there was no evidence for the independent transposition of Tc^r or of duplication in the cointegrates of the entire IS*46*(c)-Tc^r-IS*46*(d) region of N3.

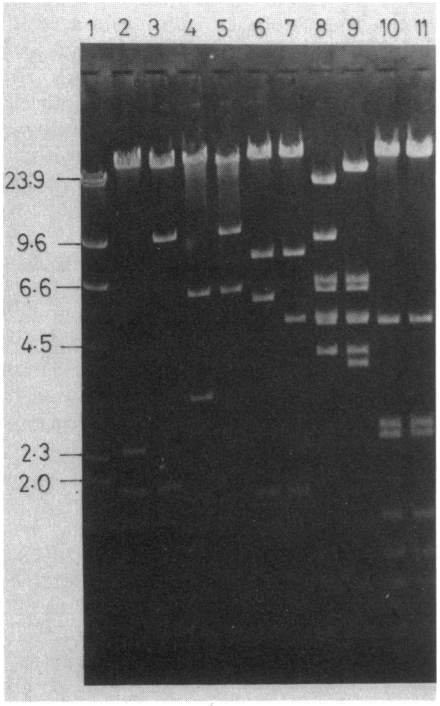

FIG. 3. Comparison of the restriction endonuclease cleavage patterns of R46A (lanes 2, 4, 6, 8, and 10) and R46B (lanes 3, 5, 7, 9, and 11) cleaved with *Hin*dIII (lanes 2 and 3), *Bam*HI (lanes 4 and 5), *Eco*RI (lanes 6 and 7), *Bgl*II (lanes 8 and 9), and *Pst*I (lanes 10 and 11). Size standard in lane 1 was produced by cleaving λ DNA with *Hin*dIII.

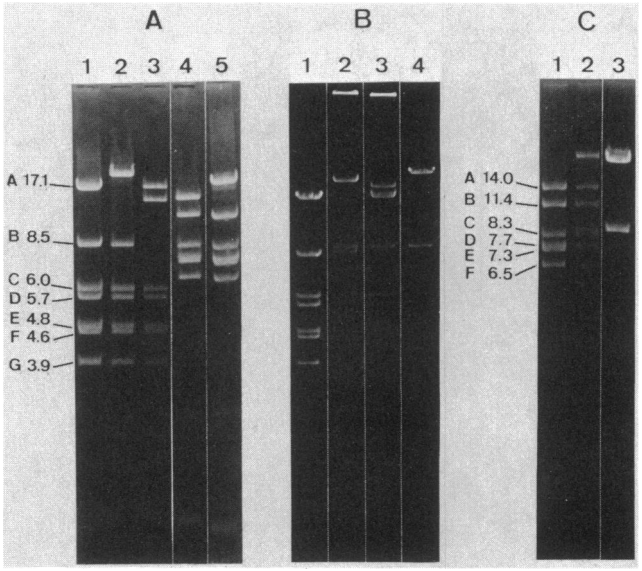

FIG. 4. *Bgl*II restriction analysis of cointegrate plasmids formed via various copies of IS*46*. (A) Lanes: 1, R46; 2, an R46::pME420 cointegrate formed via IS*46*(a); 3, an R46::pME420 cointegrate formed via IS*46*(b); 4, N3; and 5, an N3::pME420 cointegrate formed via IS*46*(d). (B) Lanes: 1, pED889; 2, a pED889::R388 cointegrate formed via IS*46*(a); 3, a pED889::R388 cointegrate formed via IS*46*(b); and 4, R388. (C) Lanes: 1, pED1017; 2, a pED1017::R388 cointegrate formed via IS*46*(c); and 3, R388. The DNA in all lanes was digested with *Bgl*II. pME420 contains no site for this enzyme, whereas R388 has two *Bgl*II sites, giving fragments of 24 and 8.6 kb. The sizes of the *Bgl*II fragments of R46 and of pED1017 (same pattern as N3) are marked in kilobases. The fragments carrying copies of IS*46* are as follows: R46 *Bgl*II-A contains IS*46*(a), R46 *Bgl*II-B contains IS*46*(b), N3 *Bgl*II-E contains IS*46*(c), and N3 *Bgl*II-A contains IS*46*(d).

In other experiments R388 mobilized the Ap^r genes of either pED1012 or pED1013 at frequencies of 5×10^{-6}. These plasmids are pBR322 derivatives containing the *Bgl*II E fragment of N3, which carries IS*46*(c), cloned in either orientation. *Bam*HI and *Sal*I digestion of plasmid DNA prepared from these transconjugants showed the patterns expected for R388::pED1012 or R388::pED1013 cointegrates resulting from IS*46*(c) transposition. IS*46*(c) transposition is therefore independent of the presence of IS*46*(d).

Resolution of R46::pME420 cointegrates. R46::pME420 cointegrates obtained as described in the previous section were used to determine whether IS*46*-mediated cointegrates could be resolved by either a plasmid- or host-encoded mechanism and consequently to provide pME420::IS*46* plasmids for studies of retransposition. Initial experiments with two cointegrates, formed via IS*46*(a) and IS*46*(b), were unsuccessful in that attempts to separate the expected Cm^r Tc^s pME420::IS*46* resolution product by either transformation or P1 transduction failed. However, it was possible that these cointegrates had been formed by R46 insertion within the replication region of pME420, so that the pME420::IS*46*

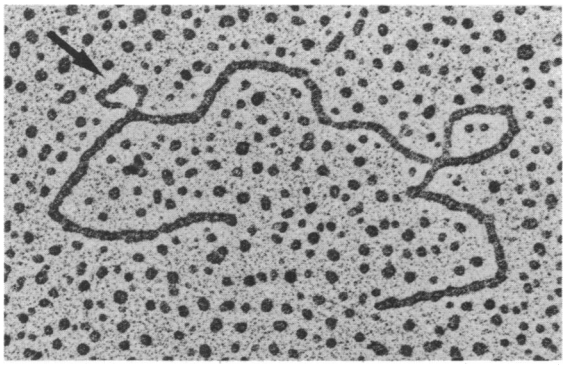

FIG. 5. A heteroduplex molecule formed between pML31 and pED994 after cleavage at their *Eco*RI sites. The 0.8-kb single-stranded loop (marked with an arrow) represents the insertion of IS*46*(d) present in pED994.

resolution product would be inviable. This had been observed previously for an IS*21*-based cointegrate (66). Because of the large size of the cointegrate molecule, it was difficult to demonstrate unequivocally that this was indeed the case, although *Pst*I digestions indicated that it was possibly so (data not shown). *Pst*I digestions of other cointegrates allowed one to be chosen (pED1030) in which integration had taken place [via IS*46*(a)] at a site ca. 2 kb from the *Pst*I site of pME420 and therefore definitely not in the replication region.

*Pst*I digestion of pED1030 DNA provided preliminary evidence that resolution was occurring in the Rec^+ host ED3818, since visual inspection of the gels indicated that those fragments derived from the expected (high copy number) pME420::IS*46*(a) component were present in greater molar amounts than those derived from the (low copy number) R46 component; this had not been observed for the other cointegrates. pED1030 was transformed into JC3272 and into the isogenic $RecA^-$ strain JC6310, and Cm^r Tc^r colonies were purified. Continued presence of the cointegrate plasmid was confirmed by showing that the antibiotic resistance markers of R46 and pME420 were transferred in filter matings at similar frequencies and that their coinheritance was 100%. Plasmid DNA was then isolated from each strain before and after growth through ca. 100 generations and was used to transform ED8654. Cm^r transformants were selected and tested for coinheritance of Tc^r, and the sizes of any Cm^r Tc^s plasmids were measured on agarose gels. There was no cointegrate resolution in the $RecA^-$ host during this growth period, whereas in the Rec^+ host the proportion of Cm^r transformants that were Tc^s increased by ca. 70%. This technique for detecting resolution has the advantage that the frequency of formation of Cm^r Tc^s transformants is increased relative to that of Cm^r Tc^r ones by the multicopy nature and small size of the pME420::IS*46*(a) resolution product; however, it has the concomitant disadvantage that the rate of resolution cannot be meaningfully quantitated. In similar experiments, R388::pED1022 (see below) was found to resolve slowly in Rec^+ but not $RecA^-$ cells.

Retransposition of IS46(a) from pED1022. One pME420::IS*46*(a) plasmid from the previous experiment was chosen, shown to have inherited *Sal*I and *Pst*I sites of IS*46*(a), and numbered pED1022. It was then transformed into JC6310 $(R388)^+$ to study the retransposition of IS*46*(a) via cointegrate formation. In conjugation experiments between this strain and the *recA*$^-$ recipient ED3886, the Cm^r marker of pED1022 was transferred at 10^{-6} of the R388 transfer frequency. Plasmid DNA was prepared from four independent Cm^r Tp^r transconjugants, and digestion with *Sal*I confirmed that in each case it gave the pattern expected for R388::pED1022 cointegrates with a copy of IS*46*(a) at each boundary.

IS*46*(a) was therefore retransposable from pED1022, demonstrating that no genes required for its transposition are located elsewhere on the R46 plasmid from which it was originally derived.

Physical characterization of IS*46*. Before pME420 became available to us, we had used pML31 as the receptor plasmid to study IS*46* transposition. pML31 is a nonconjugative, Km^r, mini-F replicon that has previously been used in Tn*3*-mediated transposition experiments (15), although the Km^r gene is present within Tn*903*, which is transposable at low frequency (48). In these experiments, an N3::pML31 cointegrate was obtained that had resulted from IS*46*(d) transposition, and P1 transduction was used to obtain the pML31::IS*46*(d) resolution product of this cointegrate. One Km^r transductant (out of 186 tested) was Spc^s Sul^s Tc^s and carried a plasmid numbered pED994. Restriction analysis showed that it was ca. 0.8 kb bigger than pML31 and carried the extra *Pst*I and *Sal*I sites characteristic of IS*46*.

Restriction analysis of pED994 showed that IS*46*(d) was located within a 4.51-kb *Eco*RI-*Hin*dIII fragment. The equivalent fragment in pML31 measured 3.7 kb, indicating that 0.81 kb of DNA had been added. This value was similar to the length of DNA duplicated in N3::pME420 cointegrates and was taken to be an accurate measure of the total size of IS*46*(d). The orientation of IS*46*(d) within the *Eco*RI-*Hin*dIII fragment was determined from double digests, using *Eco*RI together with *Sal*I or *Pst*I, and the distance from the *Eco*RI site to the *Sal*I site within IS*46*(d) was accurately measured

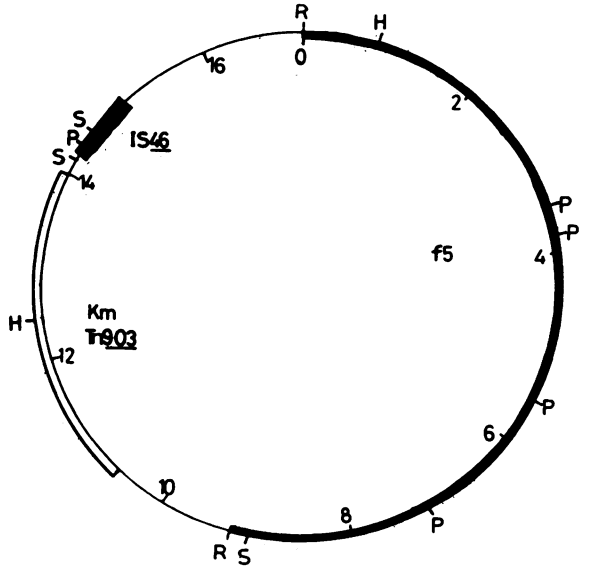

FIG. 6. A map of pED994, a pML31 plasmid carrying an insertion of IS*46*(d) (shown as a solid box). The restriction map of pML31 is based on the data of Kahn et al. (33) and Timmis et al. (60). The *Pst*I and *Sal*I sites of IS*46* are located 145 and 470 bp from the termini of the element, respectively, and the internal *Pst*I-*Sal*I distance is 195 bp. The restriction enzyme cleavage sites shown are *Eco*RI (R), *Pst*I (P), *Sal*I (S), and *Hin*dIII (H). The f5 *Eco*RI fragment derived from the plasmid F is shown as a thicker line, and the transposon Tn*903* is designated by double lines.

as 2.41 kb. The size of the internal *Sal*I-*Pst*I fragment of IS*46*(d) was 195 bp.

The distance from the same *Eco*RI site to the point of IS*46*(d) insertion was then determined by electron microscopy. Heteroduplex molecules formed between *Eco*RI-digested pED994 and pML31 DNA showed a single insertion loop corresponding to IS*46* located at 1.94 ± 0.04 kb (mean of 11 molecules) from the *Eco*RI site (Fig. 5). The distance from one terminus of IS*46*(d) to the internal *Sal*I site is, consequently, 2.41 − 1.94 = 0.47 kb, and from the internal *Pst*I site to the other terminus is 810 − 470 − 195 = 145 bp. The size of the single-stranded loop in the heteroduplexes was measured as 0.78 ± 0.02 kb (mean of 10 molecules), which is in good agreement with the size of IS*46*(d) determined from restriction enzyme digests. No double-stranded "stem" bounding this loop was visible, suggesting that if inverted repeat sequences are present at the termini of IS*46*, they are probably shorter than 100 bp. Maps of IS*46*(d) and pED994 are shown in Fig. 6.

The four copies of IS*46* show homology to each other and to IS*15* but not to IS1. To demonstrate that the four copies of IS*46* in R46 and N3 are homologous, the two plasmids were digested with *Bgl*II, and the fragments were separated on agarose gels and transferred to nitrocellulose filters (57). Duplicate filters were then hybridized to nick-translated, ^{32}P-labeled pED994 or pML31 (control) probe DNA. pED994 hybridized equally strongly to each of the four *Bgl*II fragments carrying separately IS*46*(a), IS*46*(b), IS*46*(c), and IS*46*(d), thus confirming the close similarity of all four repeated sequences (Fig. 7A).

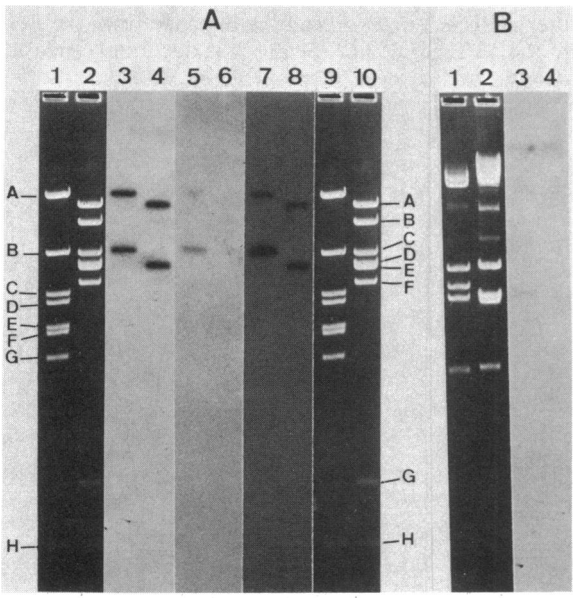

FIG. 7. (A) Gel photographs of the *Bgl*II cleavage fragments of R46 (lanes 1 and 9) and N3 (lanes 2 and 10) and autoradiographs of the same two lanes after Southern transfer and hybridization with ^{32}P-labeled pED994 (lanes 3 and 4), pML31 (lanes 5 and 6), or pIP1091 (lanes 7 and 8). The positions of the *Bgl*II restriction fragments of R46 and N3 are marked next to lanes 1 and 10, respectively. The weak hybridization of pML31 to *Bgl*II-B of R46 (lane 5) was unexpected and is not understood, whereas the stronger hybridization of pIP1091 to this fragment (lane 7) than to others containing IS*46* is due to the identical Tc^r determinants of R46 and pIP1091. (B) Gel photographs of the *Eco*RI fragments of EDλ4 (lane 1) and VAλ3 (lane 2) and autoradiographs after Southern transfer and hybridization with ^{32}P-labeled pED994.

Further hybridization experiments were carried out to demonstrate the nonhomology of IS*46* with IS*1*, an insertion sequence of similar size. First, pED994 did not hybridize to the *Eco*RI fragment of VAλ3 (EDλ4*exo*::IS*1*) that includes IS*1* (Fig. 7B). Second, ^{32}P-labeled pBR322Δ::IS*1* DNA did not hybridize to three of the four *Bgl*II fragments of R46 and N3 which carry copies of IS*46*, although it did to the fourth (data not shown). In a control experiment ^{32}P-labeled pBR322 DNA also hybridized strongly to the fourth fragment, *Bgl*II-B of R46; this fragment contains the Tc^r determinant of R46, known to be closely related to that of pBR322 (8). Strong hybridization of pBR322Δ::IS*1* to the appropriate *Eco*RI fragment of VAλ3 confirmed that IS*1* hybridization could be detected in this experiment. We therefore conclude tht IS*46* and IS*1* are not closely related.

Recently, a new insertion sequence, IS*15*Δ, has been described that is similar in size to IS*1* but does not share DNA homology with it (36, 37). Since, like IS*46*, this has single *Pst*I and *Sal*I sites, we attempted to hybridize ^{32}P-labeled pIP1091, a pBR322 derivative carrying IS*15*Δ, to *Bgl*II-digested R46 and N3. Strong hybridization to the *Bgl*II-digested bands containing IS*46* confirmed that IS*46* and IS*15*Δ are indeed related (Fig. 7A). This finding is considered further below.

Search for copies of IS*46* on other plasmids and in bacterial chromosomes. Representative plasmids from six different incompatibility groups were screened for the presence of IS*46* or related sequences by Southern blot hybridization. Restriction digests of the plasmids F (IncFI), R1-19 (IncFII), R100 (IncFII), R68 (IncP), RSF1010 (IncQ), R388 (IncW), R390 (IncN), pCU1 (IncN), and R46 (IncN) were separated by electrophoresis in agarose gels and transferred to nitrocellulose filters. These were then hybridized with the internal *Sal*I-*Pst*I fragment of IS*46*(d) ^{32}P-labeled by nick translation. With the exception of the R46 control and pCU1, none of these plasmids exhibited homology with the IS*46* probe fragment (data not shown). pCU1 contains one pair of the closely spaced *Pst*I and *Sal*I sites characteristic of IS*46* (35).

Similarly, nitrocellulose blots of *Eco*RI-digested chromosomal DNA from a variety of species of the families *Enterobacteriaceae* and *Pseudomonadaceae* were hybridized with the IS*46*-specific probe. R46 DNA was used as a hybridization control on the same filters. The species tested were *E. coli*, *Salmonella typhimurium*, *Shigella sonnei*, *Proteus morganii*, *Serratia marcescens*, *Providencia stuartii*, *Enterobacter aerogenes*, and *Pseudomonas aeruginosa*. None of these chromosomal DNA preparations showed any significant homology with IS*46* (data not shown).

DISCUSSION

We have shown that the conjugative IncN plasmids R46 and N3 each contain two copies of a sequence, designated IS*46*, which has the transposition properties of an insertion sequence. All four copies of IS*46* displayed homology in hybridization experiments and contained a common *Sal*I-*Pst*I internal fragment of ca. 195 bp; we therefore conclude that they are closely related, if not identical.

The transposition of insertion sequences results in either insertion of a discrete copy of the element within a target replicon or fusion of the donor and target replicons to form a cointegrate molecule with a copy of the element at each boundary (for a review, see ref. 34). A model whereby these two different transposition products result from diverging

alternative transposition pathways has been proposed (22, 26). Our assay of IS*46* transposition was based upon cointegrate formation and consequent conjugative mobilization either of the Tc^s pBR325 derivative pME420 by the IncN plasmid or of a *tra*$^-$ IncN plasmid mutant by the transposon-free IncW plasmid R388. Cointegrate formation took place in a RecA$^-$ host strain, as expected for an insertion sequence-based mechanism. Restriction enzyme cleavage analysis of the cointegrates showed that cointegration had occurred at a variety of sites on the recipient replicon and that a copy of IS*46* was present at each interreplicon boundary. Both findings are again characteristic of cointegrate formation via insertion sequence transposition. In addition, such analysis showed which copy of IS*46* was involved. Taking the results overall, each of the four copies of IS*46* transposed with approximately similar frequencies; these varied from 10^{-7} to 10^{-5}, according to which mobilization assay system was used. None of the other inverted repeat sequences on R46 (Fig. 1 and 2; 39) or N3 (data not shown; Coupland et al., in preparation) were found to be duplicated in any of these cointegrates, so that either these are transposable at frequencies that are one to two orders of magnitude less than that of IS*46* or are not transposable at all.

Resolution of both R46::pME420 and R388::pED1022 cointegrates was followed in Rec$^+$ and RecA$^-$ strains; it took place only in the Rec$^+$ host. In common with most other insertion sequences and in contrast to γ-δ and Tn*3* then (34), IS*46* does not encode a site-specific recombination system giving efficient resolution of cointegrate molecules. Such resolution therefore relies upon reciprocal recombination between the two IS*46* copies, brought about by the host *recA*-dependent, generalized recombination system, which is relatively inefficient for such short regions of homology (4, 23). One of the pME420::IS*46*(a) resolution products formed in a Rec$^+$ host was used to demonstrate that IS*46*(a) could be retransposed to R388, forming a cointegrate, and hence that IS*46*(a) transposition is independent of other R46 DNA sequences.

Dodd and Bennett (20) have recently described a resolution site (*res*) located on the *Pst*I F fragment of R46, which contains one terminus of IS*46*(a). However, the absence of resolution of pME420::R46 cointegrates in RecA$^-$ cells suggests that this *res* site is unlikely to be a part of IS*46*. Furthermore, pED1022::R388 cointegrates were stable in RecA$^-$ cells even in the presence of R46, showing that the R46 resolution system which will act in trans to resolve R46::Tn*3* cointegrates will not resolve cointegrates formed via IS*46*.

A further property manifested by insertion sequences is the deletion of adjacent segments of DNA. Such deletions extend outwards from the last nucleotide of the transposable element for variable distances, such that the element itself is not deleted (58). The plasmid pKM101 is an As^s Tc^s Sul^s Spc^s deletion derivative of R46 (Fig. 2) that is included in bacterial strains used for the Ames test for the detection of carcinogens as mutagens (44). Although derived by two consecutive deletion events (47), pKM101 differs from R46 by the absence of a single contiguous DNA segment of 15 kb (8, 40). One of the endpoints of this deleted region lies at, or extremely close to, IS*46*(a), and it is most likely that the deletions that formed pKM101 were generated by this element. Similarly, a fortuitously isolated transfer-deficient R46 mutant was shown to have a deletion extending into the transfer region from a point at or near IS*46*(a).

The two copies of IS*46* present on both R46 and N3 provide short regions of DNA homology across which intramolecular recombination may take place. On R46 the IS*46* elements are in opposite orientations, and recombination leads to inversion of the intervening segment, accounting for the existence of the two distinct forms of the R46 molecule designated R46A and R46B. In contrast, on N3 the IS*46* copies are directly repeated so that recombination would result in excision of the region between them. Its restriction fragment pattern showed that pED991, a spontaneous Tc^s deletion derivative of N3, is probably the product of such an excision. The loss of Tc^r from pED991 would then be analogous to the excision of resistance-determinant regions bounded by directly repeated copies of IS*1* from IncFII R factors in *S. typhimurium* and *Proteus mirabilis* (29, 62).

A number of well-characterized transposons contain insertion elements at their termini (34). The transposition functions of such "compound" transposons are encoded entirely by one or both terminal elements, which are, in most cases, independently transposable themselves. Moreover, it seems likely that any segment of DNA flanked by active insertion sequences in either orientation is potentially transposable. Examples for the short insertion sequence IS*1* include Tn*9* (42), Tn*1681* (56), Tn*2350* (11), and Tn*2571* (31). The region of R46 flanked by copies of IS*46* contains the determinants for Tc^r and As^r, whereas in N3 the intervening segment contains a Tc^r determinant. None of the transposition-mediated R46::pME420 or N3::pME420 cointegrates analyzed showed duplications of these larger regions, so we investigated the possibility that their transposition might preferentially result in simple insertions, rather than replicon fusions. No such Tc^r insertions into R338 were detected, however, and if generated at all, they must have been present at $<3 \times 10^{-9}$ per cell. Similarly, we were unable to detect transposition to R388 of the Tc^r region of N3. These results may simply reflect the long distance between the two IS*46* elements in each case: Chandler et al. (9) showed that for IS*1*-flanked composite transposons, there was a twofold decrease in the frequency of transposition for each extra kilobase of DNA between the insertion sequences. In the present case, this would give an approximately 1,000-fold reduction, so that transposition of Tc^r would be essentially undetectable.

Physical characterization of IS*46* showed that it is ca. 810 bp in length and contains single restriction sites for *Sal*I and *Pst*I that are located 470 and 145 bp from the termini, respectively. It contained no sites for *Eco*RI, *Hin*dIII, *Bam*HI, *Bgl*II, *Kpn*I, *Sma*I, or *Xho*I.

The restriction enzyme cleavage pattern plus the inability of IS*46* to hybridize to plasmids containing IS*1* (pBR322Δ::IS*1*, R100, and R1-19), IS*2* (F), IS*3* (F), γ-δ (F), IS*10* (R100), IS*21* (R68), or Tn*1* (1-19) showed that it is distinct from these transposable elements as well as from IS*4*, IS*5*, IS*50*, and IS*903* (34).

However, IS*46* seems to be related to the insertion sequences IS*15* (1.5 kb) and IS*15*Δ, IS*26*, and IS*140* (all 0.8 kb) in that all of these contain single *Sal*I and *Pst*I sites, and cross-hybridization between various members of the group has been demonstrated (7, 32, 36–38). Three composite transposons consisting of a Km^r gene flanked by direct repeats of IS*26* (Tn*2680*), IS*15* (Tn*1525*), and an IS*15*-like element (Tn*6*) have been described previously (3, 32, 36), whereas a single copy of IS*140* was adjacent to a Gm^r gene in two other plasmids (7). No association of IS*46*-like elements with Tc^r genes has been reported previously. IS*15*Δ (830 bp) results from the spontaneous loss of 670 bp of duplicated DNA from IS*15* (1,500 bp), and this loss is

associated with a reduction in the frequency of transposition from 5×10^{-5} to a frequency similar to that found for IS*46* (36). This reduction in frequency, together with the reduction due to the length of DNA between the flanking insertion elements (9; see above), is probably responsible for inability to detect transposition of Tn*2680* (J. Altenbuchner [7]) as well as the potential Tc^r transposons of R46 or N3. Transposition of Tn*1525*, flanked by IS*15* itself was measurable in a similar system.

Taken together, the above publications show that IS*46*-related elements are present on plasmids belonging to a variety of incompatibility groups, including IncA (RA1), IncC (pIP1031 and R40a), IncF (pJR62 and R124; we showed that it was not present on F, R100, or R1-19), IncI (several, including pIP112, pIP565, and R144), IncM (pIP135, pIP151, pTH1), IncT (Rts1), and IncY (pIP231), as well as on the IncN plasmids R46, N3, and pCU1. Unexpectedly, we found that IS*46* did not occur on the IncN plasmid R390, despite its similarity to other IncN plasmids and the presence of a Tc^r gene. Despite this wide distribution amongst bacterial plasmids, we were unable to demonstrate the presence of IS*46* on the chromosomes of several species of enterobacteria or *P. aeruginosa*: this contrasts to the other small insertion sequence, IS*1*, which although not found in *Pseudomonas* strains (66), is fairly widely distributed amongst the *Enterobacteriaceae* family (49, 53).

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