Embedded elements in the IncP β plasmids R772 and R906 can be mobilized and can serve as a source of diverse and novel elements

Steve Petrovskit and Vilma A. Stanisich

Department of Microbiology, La Trobe University, Victoria 3086, Australia

Correspondence

Vilma A. Stanisich
v.stanisich@latrobe.edu.au

IncP plasmids are important contributors to bacterial adaptation. Their phenotypic diversity is due largely to accessory regions located in one or two specific parts of the plasmid. The accessory regions are themselves diverse, as judged from sequenced plasmids mostly isolated from non-clinical sources. To further understand the diversity, evolutionary history and functional attributes of the accessory regions, we compared R906 and R772, focusing on the *oriV*–*trfA* accessory region. These IncP β plasmids were from porcine and clinical sources, respectively. We found that the accessory regions formed potentially mobile elements, Tn510 (from R906) and Tn511 (from R772), that differed internally but had identical borders. Both elements appeared to have evolved from a TnAO22-like *mer* transposon that had inserted into an ancestral IncP β plasmid and then accrued additional transposable elements and genes from various proteobacteria. Structural comparisons suggested that Tn510 (and a descendent in pB10), Tn511 and the *mer* element in pJP4 represent three lineages that evolved from the same widely dispersed IncP β carrier. Functional studies on Tn511 revealed that its *mer* module is inactive due to a *merT* mutation, and that its *aphA1* region is prone to deletion. More significantly, we showed that by providing a suitable transposase gene *in trans*, the defective Tn510 and Tn511 could transpose intact or in part, and could also generate new elements (stable cointegrates and novel transposons). The ingredients for assisted transposition events similar to those observed here occur in natural microcosms, providing non-self-mobile elements with avenues for dispersal to new replicons and for structural diversification. This work provides an experimental demonstration of how the complex embedded elements uncovered in IncP plasmids and in other plasmid families may have been generated.

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INTRODUCTION

Plasmids of the broad-host-range incompatibility group P (also denoted IncP-1) were first detected in *Pseudomonas aeruginosa* and enteric bacteria from clinical sources (Thomas & Smith, 1987). They are considered to be potent mediators of horizontal gene transfer, since they promote both inter- and intra-species conjugation and can replicate in taxonomically disparate proteobacteria (Adamczyk & Jagura-Burdzy, 2003; Krishnapillai, 1988; Thomas, 2000). Until the last decade, significant sequence data were available only for plasmids RP1/RK2 (the so-called ‘Birmingham’ plasmids) (Pansegrau *et al.*, 1994) and R751 (Thorsted *et al.*, 1998), which, respectively, serve as

the prototype plasmids of the IncP α and IncP β subgroups. Currently, 10 or more IncP β plasmids have been fully sequenced as have several representatives of four other IncP subgroups (Bahl *et al.*, 2009; Schlüter *et al.*, 2007; Sen *et al.*, 2010). Each subgroup contains phylogenetically distinguishable plasmid lineages (Bahl *et al.*, 2009; Kamachi *et al.*, 2006). The newer collection of IncP plasmids, in contrast to the early one (Smith & Thomas, 1989), has been drawn mainly from non-clinical sources (e.g. water, activated sludge and soil) and from diverse hosts, some unknown (Bahl *et al.*, 2009; Schlüter *et al.*, 2007). The ubiquitous distribution of the IncP plasmids underscores their importance in horizontal gene transfer and has led to efforts to define and better understand plasmid population dynamics and factors that affect them (De Gelder *et al.*, 2005, 2007).

Almost all of the IncP plasmids studied encode traits such as resistance to various antibiotics and antimicrobials, and degradation of complex compounds and xenobiotics, many of which are clinically and environmentally relevant (Bahl *et al.*, 2009; Schlüter *et al.*, 2007). In some instances, the

[†]Present address: Biotechnology Research Centre, La Trobe University, PO Box 199, Victoria 3552, Australia.

Abbreviations: DR, direct repeat; IR, inverted repeat; TE, transposable element.

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traits are encoded by class I or class II transposons that are demonstrably self-mobile (Schlüter *et al.*, 2007; Villarroel *et al.*, 1983). Such modular elements represent acquired accessory genes that have persisted on the plasmid because of a selective advantage to the bacterial host. Knowledge of the plasmid DNA sequences has been crucial in revealing the true nature and complex organization of the accessory genes, which include combinations of functional modular elements as well as defective and degraded structures and incidental (orphan) genes. In some instances, the entire accessory unit is phenotypically cryptic (Schlüter *et al.*, 2007).

The accessory genes are typically confined to two regions of the plasmid (Trefault *et al.*, 2004; Villarroel *et al.*, 1983), thereby interrupting a conserved plasmid 'backbone' that was postulated to be contiguous in the IncP plasmid progenitor (Smith & Thomas, 1987, 1989; Smith *et al.*, 1993) and is recognizable as such in some extant members [e.g. pBP136 (Kamachi *et al.*, 2006) and pJP4 (Trefault *et al.*, 2004)]. The two regions are situated between the replicative functions, *oriV* and *trfA*, and the conjugation functions, *tra* (Tra 1) and *trb* (Tra 2). The accessory genes that occur in the two regions are not necessarily located at the same site in the backbone, so that plasmids can be distinguished phylogenetically based on the position and type of insert that is present (Kamachi *et al.*, 2006; Sota *et al.*, 2007). Of relevance to this study are the IncPβ plasmids pB10 (Schlüter *et al.*, 2003), pJP4 (Trefault *et al.*, 2004), R772 (Coetzee, 1978) and R906 (Terakado & Mitsuhashi, 1974). The first two of these have been fully sequenced, and molecular maps, supported by limited sequence data, are available for R772 (~61 kb) and R906 (~58 kb) (Smith & Thomas, 1987, 1989; Smith *et al.*, 1993).

Thorsted *et al.* (1998) suggested that accessory genes may target the *oriV-trfA* and *tra-trb* regions because each region contains an array of conserved 20 bp inverted repeats (IRs) proposed to facilitate insertion of DNA segments. Although attractive, no experimental evidence to support this view was obtained in a study on the targeting of Tn21Km to the *oriV-trfA* region of pBP136 (Sota *et al.*, 2007). Instead, plasmid fitness of the insertion derivatives appeared to be a predominant factor. This finding accorded with the preponderance of Tn21-like elements found naturally in the *oriV-trfA* region of IncP plasmids generally (Schlüter *et al.*, 2007) and with the observed conservation of the plasmid backbone. A different contributing factor accounts for the predominant insertion of Tn5053/Tn402-like elements in the *tra-trb* region (Schlüter *et al.*, 2007). This interaction stems from the transpositional dependence of these elements on a target-encoded resolvase (ParA) that assists oriented and directed insertion into the cognate *res* (resolution) sequence located in the *tra-trb* region (Kamali-Moghaddam & Sundström, 2000; Minakhina *et al.*, 1999; Petrovski & Stanisich, 2010).

The main focus of this study is R772 (IncPβ), obtained from a clinical *Proteus mirabilis* strain isolated in the USA in 1974 (Coetzee, 1978). The molecular map of R772 (Hille

et al., 1983; Smith & Thomas, 1987) indicates that its accessory genes occur in the *oriV-trfA* and *tra-trb* regions and are distinctive. Those in the *oriV-trfA* region are responsible for the sole (kanamycin)-resistance phenotype of R772 (Hille *et al.*, 1983), and also include a suspected cryptic Tn501-like mercury(II)(*mer*)-resistance determinant (Smith & Thomas, 1987). The position and sequence of the *mer*-associated IR of the cryptic element have been determined (Smith *et al.*, 1993) and are identical to those of an intact Tn501-like element in pB10 and pJP4, suggesting that all three plasmids are derived from the same ancestral plasmid (Schlüter *et al.*, 2003). R906 is apparently also a member of this group that is similar to pB10, based on comparative restriction-enzyme profiling (Smith & Thomas, 1987; Schlüter *et al.*, 2003). Its accessory genes also occur in the *oriV-trfA* and *tra-trb* regions and are responsible for the multiresistance phenotype of R906. This plasmid was isolated in Japan during 1969–1972 from a porcine *Bordetella bronchiseptica* strain (Hedges *et al.*, 1974; Terakado & Mitsuhashi, 1974).

Here, we report the sequence and functional attributes of the accessory genes in the *oriV-trfA* regions of R772 and R906. Our aim was to determine the diversity and evolutionary history of the genes and their propensity for lateral transfer. Our findings support the view (Schlüter *et al.*, 2003, 2007) that the *oriV-trfA* accessory region is a mosaic of transposable elements (TEs), and that the regions in R772, R906, pJP4 and pB10 are phylogenetically related. We also observed, as have others (Schlüter *et al.*, 2007), that the earlier accrued elements may lack mobility functions due to deletion mutations. Few studies have systematically assessed the ability of the *in situ* accessory unit, and its parts, to relocate. Our findings demonstrate that relocation is possible and that the R772 unit is especially dynamic. Since accessory genes occur in other families of plasmids and are components of bacterial genomes (e.g. Kung *et al.*, 2010), our observations have broader applicability.

METHODS

Strains, plasmids and media. The auxotrophic *Escherichia coli* K-12 derivatives used were DH5α (*recA1* Res[−] Mod⁺ Nal^R) (Hanahan, 1983), LT101 (*recA13* Res[−] Mod[−] Rif^R) (Palombo *et al.*, 1989), UB281 (Rec⁺ Nal^R) (Hedges *et al.*, 1973) and its recombination-deficient derivative UB5201 (Sanchez *et al.*, 1982). The plasmids used are listed in Table 1. R772-1 has a mutation in *tnpA* of Tn5403 (*tnpA*₅₄₀₃) that was created by *Bam*HI cleavage of the purified plasmid, treatment with T4 DNA polymerase and religation. The introduced frameshift mutation is at nucleotide 2570 of *tnpA*₅₄₀₃. Additional plasmids that were generated *in vivo* are described in the text and in Table 4 (i.e. pBR322 with inserts of Tn510, Tn511, Tn511-2, Tn511-3 or Tn5403). Bacterial strains were grown at 37 °C in nutrient agar (NA) and nutrient broth (NB), as described previously (Palombo *et al.*, 1989). NA was supplemented with the required antimicrobial agents at the following concentrations (μg ml^{−1}): oxacillin (Oxa) 200; chloramphenicol (Cm), gentamicin sulphate (Gm), kanamycin sulphate (Km), mercuric chloride (Hg) and tetracycline hydrochloride (Tc) 10; ampicillin (Ap) and rifampicin (Rif) 100; nalidixic acid (Nal) 8; streptomycin sulphate (Sm) 5.

Determination of Hg^{II}-susceptibility. The sensitivity of strains was tested by agar diffusion assay. The surface of an NA plate was flood-inoculated with an overnight NB culture of the strain adjusted to 10⁸ c.f.u. ml⁻¹. A sterile 12.7 mm paper disk (Schleicher & Schuell, Antibiotica-testblattchen) was placed at the centre of the dry surface and 50 µl 10 mM HgCl₂ added to the disk. The diameter of the zone of inhibition was measured after incubation for 18 h.

Conjugation procedures, transposition assay and analysis of transconjugants. Exponential-phase cultures in NB were employed in conjugation experiments performed by the quantitative filter method (Palombo *et al.*, 1989). The donor strains (derivatives of DH5α) contained three plasmids that were sequentially introduced by transformation (for non-conjugative plasmids) or conjugation (for self-transferable plasmids), as described previously (Petrovski & Stanisich, 2010). LT101 (Rif^R) served as the recipient. The transposition assay involved the formation of cointegrates between the conjugative plasmid [i.e. R772, R772-1 (both Km^R) or R906 (Hg^R)] and the target plasmid, pBR322 (Tc^R Ap^R), in the presence of a third plasmid (Cm^R) carrying a functional or non-functional transposase (*tnpA*₂₁) gene. Since pBR322 is non-conjugative and non-mobilizable (it lacks *mob* genes), the transconjugants that are recovered on Tc-selective agar medium have inherited transpositional cointegrates. Consequently, they display the resistance profile of the two component plasmids except in instances where transposition involved the pBR322 *amp* gene. Resolution of cointegrates in the recipient was detected by recovering the pBR322 component (i.e. pBR322::Tn or pBR322*amp*::Tn). This was done by extracting DNA from Tc^R Ap^R or Tc^R Ap^S transconjugants and transforming it into *E. coli* DH5α via Tc^R selection. The resistance profile of the transformants indicated that all had inherited the resolved pBR322 component and not an unresolved cointegrate. In the case of pBR322*amp*::Tn plasmids, the insertion site and orientation of the transposon within *amp* were determined in sequencing reactions using primers SP1 (5'-CTAT-CTCAGCGATCTGTC-3'), SP2 (5'-CGCATACACTATTCTCAG-3') and SP3 (5'-TGTATCCGCTCATGAGAC-3'). The term 'α-orientation' refers to transposon insertions in which the maps of pBR322 and the transposon are similarly oriented; 'β-orientation' refers to insertion in the opposite orientation. The stable cointegrate pVS1650 was formed

during transposition of Tn510 from R906 to pBR322 (Table 4) and contains one complete copy of Tn510 and also a Tn510 remnant. The structure of pVS1650 was determined from the *Sst*I restriction profile and from analysis of the DNA sequences at each Tn510 junction in pVS1670 (Fig. 3). The sequence data were obtained using primers SP1 and SP3, and showed that Tn510 was inserted at nucleotide 3319 of the pBR322 *amp* gene and was flanked by 5 bp direct repeats (DRs) (AGATA). It also showed that one copy of Tn510 in pVS1650 had sustained an extensive deletion (from nucleotide 194 in '*tnpA1*' to IRm1; Figs 1 and 3), thereby abolishing the TnpR-*res* resolution system.

DNA techniques and sequence determination. DNA manipulations and cloning were performed using standard methods (Sambrook *et al.*, 1989). The DNA sequences of Tn510 and Tn511 were determined using suitable overlapping fragments that were cloned directly from R906 and R772 into pBluescript KS+. The fragments were sequenced at the Aggenomics Sequencing Facility (Plant Biotechnology, La Trobe University, Australia) on an Applied Biosystems ABI373 DNA analyser. The DNA extension products were generated by cycle sequencing reactions employing AmpliTaq DNA polymerase and pUC/M13 forward and reverse primers (Sambrook *et al.*, 1989). The sequence data were compiled and analysed using the suites of programs provided by National Center for Biotechnology Information (NCBI; National Library of Medicine, Bethesda, MD USA) and ExPasy (Swiss Institute of Bioinformatics, Geneva). PCR amplification of DNA (Petrovski & Stanisich, 2010) employed primers SP9 (5'-GAATTCGCTTATCGAG-CAACTCCAT-3') and SP10 (5'-GGATCCGAATTTAATCGCGGCCT-CG-3') to amplify *socD*₇₇₂, and SP32 (5'-GCGTCGACATACTTGAG-AGCGGATCAG-3') and SP33 (5'-GCGTCGACGTATCAAAGCAAG-ATCCTG-3') to detect Tn511-4 (Km^S) mutants of R772.

RNA extraction and RT-PCR. Exponential-phase cultures were grown in 10 ml NB to OD₆₀₀ ~0.3, mixed with 500 µl phenol:chloroform (5:95) and the cells were pelleted and resuspended in 500 µl ice-cold buffer [10 mM KCl, 5 mM MgCl₂, 10 mM Tris/HCl (pH 7.5)]. An equal volume of hot phenol [one part phenol and five parts buffer [0.4 M NaCl, 40 mM EDTA, 1% (v/v) β-mercaptoethanol, 1% (w/v) SDS, 20 mM Tris/HCl, pH 7.5]] was added and the total incubated at 95 °C for 1 min to lyse the cells. After centrifugation, nucleic acids in the supernatant were extracted with 300 µl phenol:chloroform (1:1)

Table 1. Plasmids used in this study

Plasmid	Relevant characteristics	Reference or source
R772	IncPβ conjugative plasmid; Tn511 ⁺ Km ^R	Coetzee (1978)
R772-1	R772 with <i>tnpA</i> ₅₄₀₃ mutation in Tn511	This study
R906	IncPβ conjugative plasmid; Tn510 ⁺ Hg ^R Oxa ^R Sm ^R Su ^R	Hedges <i>et al.</i> (1974)
pBBR1MCS-5	Broad-host-range vector; Gm ^R	Kovach <i>et al.</i> (1995)
pBR322	<i>E. coli</i> vector; Ap ^R Tc ^R	Bolivar <i>et al.</i> (1977)
pBluescript KS+	<i>E. coli</i> vector; Ap ^R	Stratagene
pUB307	IncPα conjugative plasmid (Tc ^R Km ^R)	Bennett <i>et al.</i> (1977)
pUB2401	pACYC184 <i>tet</i> ::Tn21; the plasmid (Cm ^R) carries Tn21 (Hg ^R Su ^R Sm ^R)	de la Cruz & Grinsted (1982)
pVS1650	Stable R906-pBR322 cointegrate; carries Tn510 and a Tn510 remnant; Hg ^R Oxa ^R Sm ^R Su ^R Tc ^R Ap ^S	This study
pVS1659	pBluescript KS+ with R906 <i>Eco</i> RI-HindIII clone encoding <i>merTP</i> ₅₁₀ Ap ^R	This study
pVS1660	<i>Bam</i> HI deletant of pUB2401; lacks most of Tn21 but retains <i>tnpA</i> ₂₁ ; Cm ^R	This study
pVS1661	<i>Sph</i> I-EcoRV deletant of pVS1660; lacks all of Tn21; Cm ^R	This study
pVS1670	<i>Sst</i> II-ligation derivative of pVS1650; contains pBR322 and flanking Tn510 sequences; lacks most of R906; Tc ^R	This study
pVS1683	pBluescript KS+ with R772 <i>Eco</i> RI-HindIII clone encoding <i>merTP</i> ₅₁₁ ; Ap ^R	This study
pVS1692	pBBR1MCS-5 with R906 <i>Eco</i> RI-HindIII clone encoding <i>merTP</i> ₅₁₀ Gm ^R	This study
pVS1719	pBR322::Tn511; Tc ^R Km ^R	This study

then twice with chloroform alone, and precipitated, on ice, with 2-propanol. After centrifugation for 15 min at 13 000 r.p.m. (17 000 g), the nucleic acid pellet was washed with 70 % ethanol, dried and dissolved in 30 µl diethyl-pyrocabonate-treated single-distilled H₂O (DEPC-sdH₂O) and treated with 1 U DNase I (Promega). The RNA was extracted and precipitated, as described above, resuspended in DEPC-sdH₂O and quantified spectrophotometrically. RT-PCR was performed in two steps. Reverse transcription was performed using 2 µg template RNA annealed to primer SP8 (5'-CGTAAGCATCTCGCCAAG-3') in a reaction mixture containing 200 U Moloney murine leukaemia virus reverse transcriptase (Promega), the supplied reaction buffer, 1.25 mM of each dNTP and 25 U rRNasin RNase inhibitor (Promega), in a total reaction volume of 15 µl. After incubation at 42 °C for 1 h, the reaction was terminated by heating (95 °C for 5 min) and the reverse-transcription products were subjected to PCR amplification using *Taq* DNA polymerase (Petrovski & Stanisich, 2010) and primers SP8 and SP13 (5'-GTCTGACAAAACCGTAGC-3').

Nucleotide sequence accession numbers. The accession numbers of sequences used in this study are J01749 (pBR322), Z00027 (Tn501) and AF071413 (Tn21), together with others listed in Table 2.

RESULTS AND DISCUSSION

The *oriV*–*trfA* regions of R772 and R906 contain defective derivatives of the same *mer* transposon

We determined the DNA sequences of R906 and R772 from a *Pst*I site near *oriV* to a position about 260 bp

beyond a *Hind*III site near *trfA* (Fig. 1). On analysis, each sequence revealed the presence of a defective, nested transposon embedded within plasmid sequences. The transposon in R906 was named Tn510 (13 336 bp); that in R772 was named Tn511 (18 769 bp). Both transposons were clearly related, since their respective 38 bp IR termini (IRt1 and IRm1) are identical and differ only in a few bases from those of Tn21 (Liebert *et al.*, 1999) (Fig. 2a). The 5 bp DR sequences that flank Tn510 and Tn511 were also identical (i.e. TGCCT), and are each part of longer sequences recognizable as IncPβ backbone (i.e. the 40 bp from the *Pst*I site to IRt1 and the 262 bp beyond IRm1) (Fig. 1). A simple explanation of these features is that Tn510 and Tn511, although different in size, are both derived from a progenitor transposon that entered a common ancestor of R906 and R772 by transpositional insertion. From the parts of the Hg^{II}-resistance (*mer*) and transposition (*tnp*) modules that have been retained, the progenitor is most like the Tn21/Tn501-family transposon TnAO22 (Ng *et al.*, 2009) (Fig. 2b, c), and will be ascribed this name to aid discussion.

Specifically, the type 6 *mer* modules (*merRTPADEorf2*) of Tn510 and Tn511 are of identical length and sequence (>99 %) to those of TnAO22 and Tn501 (Liebert *et al.*, 1997) (Table 2). The *tnp* modules are incomplete. They too are near identical to *tnp* of TnAO22, and more similar to

Table 2. Features and coding sequences of Tn510 and Tn511, and their closest relatives

Feature and/or gene sequence	Tn coordinates (nucleotides) [†]		Percentage nucleotide identity to the most closely related sequences [‡]	GenBank accession numbers
	Tn510	Tn511		
IRt1-' <i>tnpA</i> 1\$	1–273	1–241	100 % to pAO22 from <i>Achromobacter</i> sp. and pMOL30 from <i>Cupriavidus metallidurans</i>	EU696790; CP000354
IS1071	274–3477	NA	100 % to pJP4 from <i>Ralstonia eutropha</i> and pTSA from <i>Comamonas testosteroni</i>	AF311437; CP000541
' <i>tnpA</i> ' _{1071-like}	NA	363–638	100 % to pCNB1 from <i>Comamonas</i> sp. and to <i>Ralstonia pickettii</i>	CP001644; AB266144
IRt2-' <i>tnpA</i> 2	NA	639–3420	99 % to pMOL28 from <i>C. metallidurans</i> and a fosmid clone from an unknown organism	AB266144; AJ877225
'Tn5393c	3478–7836	NA	99 % to pRAS2 from <i>Aeromonas salmonicida</i> and pPSR1 from <i>Pseudomonas syringae</i>	AF262622; AY342395
Tn5403	NA	3421–7083	99 % to <i>Klebsiella pneumoniae</i> and pEC_L46 from <i>E. coli</i>	X75779; GU371929
<i>socD</i> -like	NA	7442–8560	71 % to <i>Pantoea vagans</i> C9-1 and 66 % to <i>Erwinia billingiae</i> Eb661	CP002206; FP236843
<i>aphAI</i>	NA	8928–9743	98 % to pSe-Kan from <i>Salmonella enterica</i> and pRts1 from <i>Proteus vulgaris</i>	GQ426885; AP004237
IRt3-' <i>tnpA</i> 3*R	NA	10 562–14 252	99 % to pMOL28 from <i>C. metallidurans</i> and a fosmid clone from an unknown organism	AB266144; AJ877225
<i>tnpA</i> 1R- <i>orf2</i> - <i>mer</i> -IRm1	7837–13 346	14 253–18 769	99 % to pAO22 from <i>Achromobacter</i> sp. and pMOL28 from <i>C. metallidurans</i>	EU696790; X90708

[†]The sequences of Tn510 and Tn511 are available under GenBank accession numbers DQ471307 and EU287476, respectively. NA, Not applicable.

[‡]The sequence of Tn510 is identical to the corresponding parts of a Tn510 derivative in pB10 (accession number AJ564903).

\$A' symbol indicates the part of the gene (or feature) that is missing (i.e. the 3' end and/or the 5' end).

^{||}These sequences are the best of only several database entries with high identity. All other sequences listed in Table 2 had high identity to numerous database entries for different plasmids and/or bacteria.

Fig. 1. Comparison of the genetic maps and mosaic structures of Tn510 (13 336 bp) and Tn511 (18 769 bp). These related defective transposons occur at the same position in the *oriV*–*trfA* backbone region of R906 and R772, respectively, bounded by identical 5 bp DRs (TGCCT). The various genes and their direction of transcription are represented by arrows. Other details are provided in Table 2. Genes and other components that are part of the same functional module are similarly shaded, except for the *tnpA*-defective Tn5393c and the orphan genes *socD* and *aphA1*, which are unshaded. IRs that flank modular elements are represented by vertical lines. The Tn21-like IRs that flank *mer* genes (IRm) or *tnp* genes (IRt) are numbered to identify the different remnant elements in which they occur. Black ovals indicate the position of resolution sites (*res*). The bars below the map of Tn511 indicate the compositions of Tn511-2 (flanked by IRt2 and IRm1), Tn511-3 (flanked by IRt3 and IRm1) and Tn511-4. The last arose from RecA-mediated recombination between *'tnpA2* and *tnpA3* at unknown sites within the region indicated by the dashed line. Black arrows with an SP number indicate the binding regions of PCR primers referred to in the text. Cleavage sites are shown for the restriction enzymes *EcoRI* (E), *BamHI* (B), *HindIII* (H), *PstI* (P), *NcoI* (Nc) and *NheI* (Nh).

the module in Tn21 (~97 % identity) than to that in Tn501 (~70 % identity). Instead of the expected *tnpAR-res* components, Tn511 contains a 3' remnant of the transposase gene (i.e. the 207 bp *'tnpA1*) adjacent to IRt1. In Tn510, the wild-type *tnpA1* has been disrupted and the central portion deleted. Consequently, the 3' remnant occurs near IRt1 (i.e. the 239 bp *'tnpA1*), and the 5' remnant (i.e. the 264 bp *tnpA1'*), together with the resolvase gene (*tnpR*) and *res* sequence, occurs near the *mer* module. It appears, therefore, that the TnAO22 *tnp* module underwent significant changes during its evolution into Tn510 and Tn511 (discussed below), whereas the *mer* module remained largely unaffected. The latter finding was unexpected, since Hg^{II}-resistance is a trait of R906 but not of R772, which is suspected to carry a *mer* remnant (Smith & Thomas, 1989). We investigated the basis of this phenotypic difference using *E. coli* K-12 as the host.

R772 confers sensitivity to Hg^{II} because the MerT import protein encoded by Tn511 is defective

The *mer* modules of Tn510 (from R906) and Tn511 (from R772) were found to be identical, except for single-base differences in *merT* (351 bp), which encodes an Hg^{II}-transport protein, and *merA* (1686 bp), which encodes the Hg^{II} reductase (Barkay *et al.*, 2003). The differences in *merT*₅₁₁ (T→A at nucleotide 32) and *merA*₅₁₁ (A→T at nucleotide 1661) result in predicted amino acid substitutions (i.e. Leu¹¹→Pro in MerT₅₁₁, Lys⁵⁵⁴→Met in

MerA₅₁₁). Either of the mutated genes could be responsible for the Hg^S phenotype of R772, since a *merA* or *merT* mutation can result in Hg^{II} sensitivity (Hamlett *et al.*, 1992). To individually assess the functions of *merT*₅₁₁ and *merA*₅₁₁, we took advantage of the fact that a different phenotype, Hg^{II} hypersensitivity, arises when wild-type *merT* is expressed in the absence of wild-type *merA* (whether or not *merP* is present) (Lund & Brown, 1987). We therefore cloned *merTP* from Tn510 and Tn511 and used disk-diffusion assays to study the phenotypic effect of the genes when present alone or in strains with R906 (which has a functional *merA*₉₀₆) or R772 (which has a potentially dysfunctional *merA*₇₇₂).

The data obtained (Table 3) confirm that R906, and not R772, confers Hg^{II} resistance (lines 1–3). They also showed that the R906 *merTP*₅₁₀ genes cause Hg^{II} hypersensitivity when *merA*₉₀₆ is absent (line 4), and that hypersensitivity was counteracted when *merA*₉₀₆ was present. The effect was partial (line 7) when Hg^{II} import was elevated, and complete (line 9) when import was lower (i.e. when the high- and low-copy-number pBlue-*merTP*₅₁₀ and pBBG-*merTP*₅₁₀ plasmids were present, respectively). Significantly, the cloned R772 *merTP*₅₁₁ genes failed to confer Hg^{II} hypersensitivity (compare lines 4 and 5). Since *merP*₅₁₀ and *merP*₅₁₁ are identical, this observation provides genetic evidence that *merT*₅₁₁ is defective. In contrast, the R772 *merA*₅₁₁ gene, despite a missense mutation, appears to be functional, since strains with the active MerTP₅₁₀ import system and with either *merA*₅₁₀ (in R906)

Fig. 2. Tn21-related IR sequences (a) and the proposed genesis of Tn510 (b) and Tn511(c). The sequences of the IR termini of Tn510 and Tn511 are compared with those of Tn21, TnAO22 and internal IRs in Tn511 (see Fig. 1 and text); each group of sequences is identical, except for the four nucleotides shown. Tn510 and Tn511 are each built on a TnAO22 platform whose genetic map is shown in black type. Transposons have been added sequentially to form Tn510 and Tn511 (see text for details). Maps of the elements are shown above the TnAO22 map. In Tn511, the order of acquisition of *socD*–*aphA1* and the *mer* transposon shown below the TnAO22 map is unknown. IRs are represented by vertical bars at the termini of the elements and *res* sequences by filled circles. Deleted sequences are indicated by dashed lines.

Fig. 3. Comparison of the structures of a Tn510-mediated transpositional cointegrate and the stable derivative pVS1650. The upper map depicts part of a typical R906–pBR322 transpositional intermediate. The pBR322 replicon (*tet*⁺ *oriV*⁺) is flanked by direct copies of Tn510 (IS1071⁺ *mer*⁺) whose left and right IRs are represented by small white and black boxes, respectively. A deletion (Δ) of most of the left copy of Tn510 resulted in the formation of pVS1650, depicted in the lower map. The structure of pVS1650 was deduced from restriction enzyme profiles (not shown) and from the DNA sequences at each pBR322–Tn510 junction present in pVS1670 using primers SP1 and SP3. The left junction contains a Tn510 remnant (nucleotides 1–194). The potentially mobile novel transposon that encompasses the pBR322 replicon was denoted Tn510-2. Cleavage sites for *SstII* (S) are indicated.

or *merA*₅₁₁ (in R772) are phenotypically similar (compare lines 7 and 10 and lines 9 and 12). Our conclusion that MerT₅₁₁ is defective is also consistent with the basal level sensitivity (i.e. a 3 mm zone) exhibited by the strain with *merTP*₅₁₁ and *merA*₅₁₁ (line 11). The changed residue in MerT₅₁₁ (Leu¹¹→Pro) is near the first putative transmembrane helix of the protein (residues 12–34) (Barkay *et al.*, 2003). Consequently, it may alter the membrane topology or function of MerT₅₁₁, thereby affecting critical residues in the helix, such as the vicinal Cys²⁴ and Cys²⁵, which are required for the Hg^{II} resistance and/or hypersensitivity phenotypes (Hobman & Brown, 1996).

Tn510 in R906 and Tn511 in R772 contain different nested components

Although Tn510 and Tn511 are clearly derived from the same TnAO22 base element, they differ in other structural components. Tn510 contains part of Tn5393c (L'Abée-Lund & Sørum, 2000): the streptomycin-resistance (*strAB*) arm is present but the 3' end of *tnpA*₅₃₉₃ and the associated IRt are missing (Fig. 1). Tn510 also contains IS1071; however, this is not flanked by the 5 bp DRs typically associated with its transposition (Sota *et al.*, 2006). Since the sequences of the Tn510 components directly abut each other, we conclude that TnAO22 first inherited Tn5393c,

Table 3. Hg^{II}-resistance phenotype of *E. coli* strains carrying cloned *merTP* genes from Tn510 and Tn511

Line number cited in text	Plasmids in <i>E. coli</i> DH5 α		Zone diameter† (mm)
	IncP β (Tn)	Coresident plasmid*	
1	None	None or empty vector	4
2	R906 (Tn510)	None or empty vector	0
3	R772 (Tn511)	None or empty vector	3
4	None	pBlue- <i>merTP</i> ₅₁₀	11
5	None	pBlue- <i>merTP</i> ₅₁₁	5
6	None	pBBG- <i>merTP</i> ₅₁₀	6
7	R906 (Tn510)	pBlue- <i>merTP</i> ₅₁₀	6
8	R906 (Tn510)	pBlue- <i>merTP</i> ₅₁₁	0
9	R906 (Tn510)	pBBG- <i>merTP</i> ₅₁₀	0
10	R772 (Tn511)	pBlue- <i>merTP</i> ₅₁₀	7
11	R772 (Tn511)	pBlue- <i>merTP</i> ₅₁₁	3
12	R772 (Tn511)	pBBG- <i>merTP</i> ₅₁₀	0

*The empty vectors were pBlue (=pBluescriptKS+) and pBBG (=pBBR1MCS-5). Derivatives of these were pVS1659 and pVS1692 (both with *merTP*₅₁₀) and pVS1683 (*merTP*₅₁₁).

†The results shown are from a single disk-diffusion assay. The data from independent assays displayed the same trends, although the diameter of zones of clearing may have differed (± 1 mm).

which then inherited IS1071 (Fig. 2b). If the respective insertions occurred towards the 5' end of *tnpA1* and the 3' end of *tnpA*₅₃₉₃, a leftward IS1071-mediated deletion would account for the missing sequences (i.e. *tnpA*'-IRt₅₃₉₃, '*tnpA1*' and the left DR of IS1071).

Tn511 is structurally more complex than Tn510. In addition to the TnAO22 base element, remnants of two other Tn21-like elements are present that differ in sequence identity. The larger remnant (IRt3-*tnpA3**-*tnpR*) probably originated from a Tn21-like element (84–89% identity) that inserted into *tnpA1* of TnAO22, and was followed by TnpR-mediated resolution at the heterologous *res* sites. Consequently, IRt3-*tnpA3**-*tnpR* forms part of a hybrid transposon that has, as its other arm, *res-mer*-IRm1 (95–99% identity to Tn21) (Fig. 1). The second Tn21-like remnant is IRt2-'*tnpA2*' (99% identity). This remnant abuts Tn5403, a Tn3-related transposon (Rinkel *et al.*, 1994a). Significantly, Tn5403 lacks its typical 5 bp DRs (Rinkel *et al.*, 1994a), suggesting that it was responsible for deleting the remainder of *tnpA2*. Since Tn5403 is the sole intact element in Tn511, we assume that it is the most recently acquired entity.

The remaining components in Tn511 include three 'orphan' genes. One is an IS1071-like remnant gene, '*tnpA*', that is in a similar position to the intact *tnpA*₁₀₇₁ in Tn510 but is only distantly related (69%); a sequence identical to '*tnpA*' occurs in an IS1071-like element in pCNB1 (IncP β) (Ma *et al.*, 2007). The other 'orphan' genes occur in a 3478 bp region that abuts Tn5403 on its right. The region is not related to reported sequences, except for *aphAI* and a *socD*-like gene (Table 2). The former encodes an aminoglycoside 3'-phosphotransferase responsible for

the Km^R phenotype of R772. The *socD*₅₁₁ product is 46% identical to an amino acid oxidase (SocD) involved in santhopine catabolism in *Agrobacterium tumefaciens* C58 (Baek *et al.*, 2005) and to putative fructosyl amino acid oxidases in other soil-dwelling bacteria (Table 2). It is possible that *socD*₅₁₁ is functional, since its transcription from R772 in *E. coli* DH5 α was detected by RT-PCR (see Methods) (data not shown). R772 is to our knowledge the first example of an IncP plasmid to carry a *socD*-like gene.

A possible model that explains the structural origin of Tn511 is depicted in Fig. 2(c).

The provision of *tnpA*₂₁ in trans enables transposition of Tn510, Tn511 and the components Tn511-2 and Tn511-3

Based on *in silico* findings we concluded that Tn510 and Tn511 are defective transposons, since they lack a complete *tnpA* gene (Fig. 1). They do, however, have intact *tnpR-res* loci (near *merE*) and terminal IRs. Thus, whilst appearing to be permanently embedded structures, we thought that Tn510 and Tn511 might relocate if a cognate *tnpA* gene (e.g. *tnpA*₂₁ from Tn21) was provided *in trans*, as might smaller sections of Tn511 if its internal IRt2 and IRt3 sequences formed compatible termini with IRm1. The intact IS1071 and Tn5403 elements were potentially self-mobile. These various suppositions were tested in conjugation experiments designed to detect transposition of Tn510 (Hg^R Sm^R), Tn511 (Km^R) and its components, in the presence and absence of *tnpA*₂₁ [on pVS1661 (Cm^R)]. The non-conjugative plasmid pBR322

Table 4. Independent and *TnpA₂₁*-assisted transposition of Tn510, Tn511 and their component elements to the target plasmid pBR322

Line number cited in text	Plasmids in donor* with target plasmid pBR322		Transconjugants†	
	IncP plasmid (Tn)	Genotype of helper plasmid	Transposition frequency	Transposition derivative (number)‡
1	R906 (Tn510)	<i>tnpA₂₁</i> [−]	$<5.0 \times 10^{-8}$	—
2	R906 (Tn510)	<i>tnpA₂₁</i> ⁺	7.0×10^{-6}	pBR322 <i>amp</i> ::Tn510 (3); pBR322 <i>amp</i> -R906 cointegrate (1)
3	R772 (Tn511)	<i>tnpA₂₁</i> [−]	2.5×10^{-6}	pBR322::Tn5403 (3); pBR322 <i>amp</i> ::Tn5403 (1)
4	R772-1 (Tn511)	<i>tnpA₂₁</i> [−]	$<2.0 \times 10^{-7}$	—
5	R772-1 (Tn511)	<i>tnpA₂₁</i> ⁺	8.8×10^{-7}	pBR322::Tn511-3 (10); pBR322::Tn511-2 (8); pBR322::Tn511 (1)

*Donors were derivatives of *E. coli* DH5 α carrying a conjugative plasmid [R906 (Hg^R), R772 (Km^R) or R772-1 (with a defective Tn5403 in Tn511)], together with pBR322 (Tc^RAp^R) and either a *tnpA₂₁*⁺ plasmid (pVS1661) or its *tnpA₂₁*[−] derivative (pVS1660) (both Cm^R). The recipient was LT101 (Rif^R).

†Transconjugants were isolated on NA containing Rif and either Hg^{II} or Km [to detect transfer of R906 and R772 (or R772-1), respectively] or Tc (to detect conductive transfer of pBR322). The transposition frequency represents the ratio of Tc^R to Hg^R transconjugants per R906⁺ donor, or Tc^R to Km^R transconjugants per R772⁺ (or R772-1⁺) donor. The mean transfer frequencies of R906 and R772 (or R772-1) were 4.0×10^{-1} and 4.2×10^{-1} , respectively. Experiments were performed at least three times using independently constructed donors.

‡The pBR322 transpositional derivatives present in transconjugants were isolated and characterized as described in Methods. The transconjugants studied were all randomly chosen from amongst the Tc^RAp^R subgroup or Tc^RAp^S subgroup.

(Tc^R Ap^R) served as the transpositional target. Its transfer was monitored via the incidence of Tc^R transconjugants and is dependent on the formation of transpositional cointegrates (see Methods). These can be resolved in the recipient by the cognate *TnpR-res* system, presuming that it is functional.

The data obtained (Table 4) show that despite efficient conjugal transfer of R906 and R772 ($\sim 4.0 \times 10^{-1}$ per donor; see footnote), only the R772⁺ donor, and not that with R906, elicited transfer of pBR322 (lines 1 and 3). The R772-mediated events were attributed to Tn5403, since none occurred in the corresponding experiment involving R772-1, a *tnpA₅₄₀₃* mutant (line 4). Confirmation of Tn5403 transposition was obtained by analysing four Tc^R transconjugants (see Methods). All contained resolved pBR322::Tn5403 plasmids in which the insert was at a different site. In one of these (from a Tc^R Ap^S transconjugant), Tn5403 was in *amp* of pBR322 (at nucleotide 4181), where it was flanked by 5 bp DRs (CATTT) and was able to relocate to pUB307 when tested further (data not shown). We concluded that Tn5403 of Tn511 is self-mobile and that its transposition to random target sites involves the formation of a cointegrate, a feature previously attributed to this element (Hille *et al.*, 1983; Rinkel *et al.*, 1994a). The failure of R906 to similarly transfer pBR322 (line 1) demonstrated that IS1071 of Tn510 is not self-mobile in this assay system, despite its reported ability to form transpositional cointegrates (Sota *et al.*, 2006; Wyndham *et al.*, 1994), and neither is Tn510 itself.

Line 2 of Table 4 shows that *tnpA₂₁* facilitated transfer of pBR322 by R906, presumably by restoring transposition of Tn510. This conclusion was confirmed by analysing four Tc^R Ap^S transconjugants, three of which contained resolved pBR322*amp*::Tn510 plasmids whose Tn510 insertion sites and orientation in *amp* were determined (i.e. at nucleotides 3710 and 4062 in β -orientation, at nucleotide 3530 in α -orientation). The fourth plasmid (pVS1650) was an unusual R906-pBR322 cointegrate that had formed by Tn510 insertion into *amp*, but had not resolved because most of the second copy of Tn510 was absent (see Methods) (Fig. 3). The structural features of pVS1650 are interesting, as the pBR322 replicon is fortuitously enclosed by IRm1 and IRT1 termini, forming the novel pBR322-Tn510 transposon Tn510-2 (Fig. 3). Although not formally tested, Tn510-2, like Tn510, should be capable of *tnpA₂₁*-assisted transposition. The IRm1 and IRT1 termini of Tn510 were identified in all four plasmids studied and in each case they were flanked by different 5 bp DRs, consistent with transposition of a Tn21/Tn501-like element.

We also studied *tnpA₂₁*-assisted transposition of Tn511 by using the Tn5403-inactive plasmid R772-1. Transposition was detected (line 5), and the resulting products in 19 Tc^R transconjugants were analysed further. All had recoverable pBR322::Tn plasmids that contained different parts of Tn511. Those from 10 Tc^R Km^S transconjugants were resolved pBR322::Tn511-3 plasmids. All were of similar size (~ 12.5 kb) and sequence analysis of four confirmed the presence of Tn511-3 (i.e. at nucleotides 2431, 2510

and 3162 in α -orientation, and at nucleotide 3143 in β -orientation). In each instance, the Tn511-3 insert was bounded by IRT3 and IRm1, and flanked by 5 bp DRs. Larger plasmids (~22.5 kb) were recovered from all nine Tc^R Km^R transconjugants. One was identified as a pBR322::Tn511 plasmid and eight as pBR322::Tn511-2 plasmids, based on the presence or absence of the *Nco*I site located near IRT1 (Fig. 1). The latter group was further divisible into four (via *Hind*III profile), suggesting differences in either the position or the orientation of the inserted Tn511-2.

These various observations provide experimental evidence of bona fide transposition of Tn510 and Tn511, and of their components (Tn511-2, Tn511-3 and Tn5403), from their *in situ* locations in R906 or R772. With the exception of Tn5403, transposition of these otherwise immobile elements was assisted by *tnpA*₂₁ supplied *in trans*. This feature is expected to extend to Tn510-2, a novel transposon that contains the pBR322 replicon. Our failure to detect transposition of IS1071 is consistent with a recent study (Sota *et al.*, 2006) which showed that transposition is host-dependent and does not occur in *E. coli* as previously suspected.

The *aphAI* region of Tn511 is inherently unstable

The DNA sequence of Tn511 revealed that *aphAI* is flanked by similarly oriented regions of homology of >2 kb in length (i.e. *tnpA*2 and *tnpA*3*) (Fig. 1). These regions are potential substrates for the host recombination (RecA) system, and could result in deletion of the intervening ~10 kb region containing Tn5403, *socD* and *aphAI*. To test this possibility, PCRs were performed using primers SP32 and SP33 (Fig. 1) and template DNA from Rec⁺ and Rec⁻ strains that had been grown for ~25 generations in the absence of kanamycin. The strains carried either R772 or pBR322::Tn511 (pVS1719). PCR products were obtained only from the Rec⁺ sources and were of ~2.5 kb in size (Fig. 4), consistent with the presence of RecA-generated plasmid deletants involving 'tnpA2 and tnpA3*. The greater product yield from the pBR322::Tn511 sample was attributed to the increased chance of deletions due to the smaller size and high copy number of this plasmid compared with R772. Indeed, ~30 % of the colonies grown from the Rec⁺ pBR322::Tn511⁺ culture were Km^S, whereas no segregants were detected (<1 %) from the Rec⁺ R772⁺ culture. The extracted plasmids from four Km^S segregants were all smaller (~10 kb) than pBR322::Tn511 and differed from it in *Hind*III and *Eco*RI profiles, consistent with loss of the Tn5403-*socD*-*aphAI* segment (Fig. 1). These observations demonstrate the emergence of another new element from Tn511, denoted Tn511-4 (Fig. 1), that, like Tn511-3, has no associated phenotype. They also imply that the maintenance of R772 in its clinical strain (Coetzee, 1978), and since then in laboratory strains, was due to aminoglycoside selection pressure. Indeed, the latter may be a strict requirement, since expression of *aphAI* imposes a cost burden on its bacterial host (Kim *et al.*, 2006).

Hypothetical genesis of Tn510 and Tn511 and their evolutionary potential

The DNA sequences of Tn510 (from R906) and Tn511 (from R772) and the experimental features of these elements provide clues to their origins. A simple scenario is that Tn510 and Tn511 evolved mainly by sequential acquisition of TEs and accompanying DNA rearrangements. In the primary event, TnAO22 inserted into the IncP β *oriV*-*trfA* region, forming the extant 5 bp target duplication. Other TEs were then acquired, altering TnAO22 internally and establishing the Tn510 and Tn511 lineages (Fig. 2). A derivative of Tn510 occurs in pB10(IncP β) (Schlüter *et al.*, 2003). Its sequence and location are identical to those of Tn510, except that IS1071 is disrupted by a Tn1721-like element. A third lineage is represented by the element in pJP4 (Schlüter *et al.*, 2003; Trefault *et al.*, 2004): it retains the *mer*-IRm1 end of TnAO22 but the IRT end and adjacent backbone sequences have been replaced by the acquired TEs. An alternative scenario is that TnAO22 evolved prior to its transposition into the same *oriV*-*trfA* site by a *tnpA*-assisted process (Table 4). This scenario is unlikely, since target specificity is not a feature of Tn21/Tn501-like elements related to TnAO22 (Grinstead *et al.*, 1990), although they favour the IncP β *oriV*-*trfA* region (Sota *et al.*, 2007).

The various components of Tn510 and Tn511, except for the regions flanking *socD* and *aphAI*, are recognizable genes or remnant TEs. We speculate that all may have been

Fig. 4. Instability of the Tn5403-*socD*-*aphAI* region of Tn511 in *E. coli*. Plasmid DNA isolated from RecA⁺ (UB281) and RecA⁻ (UB5201) strains carrying either pBR322::Tn511 (pVS1719) or R772 was PCR-amplified using primers SP32 and SP33, and the reaction products were separated by electrophoresis on an agarose gel. Lanes: 1, λ *Hind*III-digested marker DNA; 2, pVS1719⁺ strain; 3, R772⁺ strain; 4, pVS1719⁺ strain; 5, R772⁺ strain. The arrow indicates the position of a faint band that occurs in lane 5.

acquired from environmental bacteria given the ecology of the components. TnAO22 was detected in an *Achromobacter* sp. (Ng *et al.*, 2009); however, the broader family of Tn21/Tn501-like elements is common in Gram-negative bacteria (Grinsted *et al.*, 1990; Liebert *et al.*, 1999; Yurieva *et al.*, 1997). Tn5393 is typically associated with phytopathogens [e.g. *Erwinia* and *Pseudomonas* species (Chiou & Jones, 1993; Sundin *et al.*, 1995)], and IS1071 with pollutant-degrading bacteria [e.g. *Comamonas* and *Pseudomonas* species (Di Gioia *et al.*, 1998; Wyndham *et al.*, 1994)], including those that support IS1071 transposition (Sota *et al.*, 2006). Tn5403 was detected in aquatic enterobacteria [i.e. *Klebsiella* spp. and *Enterobacter agglomerans* (Rinkel *et al.*, 1994a, b)], and *socD* genes occur in members of the *Rhizobiaceae* (Baek *et al.*, 2005). The *aphAI* gene may have been from bacteria associated with humans or animals (Vakulenko & Mobashery, 2003), and is possibly a TE remnant. Alternatively, the Tn511 *aphAI-socD* region may have been inherited *en bloc* by an unknown capture process. An agrobacterial/rhizobial source is possible, since kanamycin resistance occurs amongst these bacteria (Brockman & Bezdicek, 1989; Cole & Elkan, 1979; F. Taner & V. A. Stanisich, unpublished data).

All of the above-mentioned bacteria could have been accessed by the ancestral IncP β plasmid, since the IncP family in general has a broad host range (Sen *et al.*, 2010; Thomas & Smith, 1987) and occurs in diverse habitats (Bahl *et al.*, 2009; Smalla *et al.*, 2000). Tn510 and Tn511 have added interest, since R906 and R772 were isolated in the 1970s from vertebrate sources, yet their components, apart from Tn5403 and *socD*₅₁₁, are similar to those in environmentally sourced IncP plasmids. Some of the components (e.g. Tn5393 and Tn5403) appear to be disseminating more widely and have been found in complexes with other TEs or resistance genes in bacteria from humans (Boyd *et al.*, 2004; L'Abée-Lund & Sørsum, 2000; Mantengoli & Rossolini, 2005; Ruppé *et al.*, 2009). The compositions of Tn510 and Tn511 thus lend support to the opinion that gene flow from environmental microcosms can affect bacteria present in humans (the source of R772) and animals (the source of R906) (Davies, 1994; Mindlin *et al.*, 2006; Schlüter *et al.*, 2007).

The genesis models (Fig. 2) also posit that the various components were acquired at different times, possibly as intact TEs, and were subject to mutational change. A missense mutation in *merT*₅₁₁ (Table 3) accounts for the Hg^{II} sensitivity of R772, despite its full-length *mer* module. Deletion mutations caused either by the TEs or by homology (RecA)-dependent processes are also evident. TE-mediated events are expected to leave the TE intact, whilst removing the DR and adjoining DNA on one flank (Petrovski & Stanisich, 2010; Wang *et al.*, 1994). This type of event explains why Tn5403 and IS1071 each border a remnant gene and lack recognizable DRs, even though these are reinstated on subsequent transposition, as observed for Tn5403 (Table 4). Presumably, both elements could continue to initiate deletions, producing new

variants. RecA-dependent deletions were not recognizable in Tn510 and Tn511; however, such events involving the Tn511 '*tnpA2* and *tnpA3*' sequences account for the kanamycin-sensitive derivative Tn511-4 (Figs 1 and 4). A similar process in the Tn510 derivative of pB10 could result in tetracycline-sensitive segregants.

To this point we have focused on the evolution of Tn510 and Tn511 in their IncP β carrier. Other evolutionary consequences attended transposition of the elements when assisted *in trans* by *tnpA* of Tn21. The TnpA₂₁ transposase is relatively non-specific (Grinsted *et al.*, 1990) and was able to recognize IRT1 and IRTm1, which differ from the Tn21 termini, as well as the internal IRT2 and IRT3, which do not (Fig. 2a). Consequently, transposition of Tn510 and Tn511 was detected, and also that of Tn511-2 and Tn511-3 (Fig. 1, Table 4). Similar events enabling defective elements to escape from their carrier replicon could presumably occur during natural encounters of IncP plasmids and Tn21/Tn501-like transposons in the diverse habitats in which both occur (Smalla *et al.*, 2000). One of the Tn510 transposition events resulted in the formation of an R906-pBR322 cointegrate that was stabilized by the absence of a complete second copy of Tn510 (Table 4, Fig. 3). A similar stable cointegrate was formed between R772 and pTi in *Agrobacterium*, involving Tn5403 as the initiating element (Hille *et al.*, 1983). Such cointegrates are presumably due to errors in the transposition process and may be relatively common. They enable the transfer of 'host-limited' genes (e.g. pBR322) to the pantheon of IncP hosts, where they may remain linked to the IncP replicon or have opportunities to separate from it, as predicted for the hybrid transposon Tn510-2 (Fig. 3).

In overview, this study of Tn510 and Tn511 shows how complex embedded structures may have arisen. We focused on the functional attributes of these elements to show that they are not genetic dead-ends but can contribute to horizontal gene transfer in perhaps unanticipated ways. Similar elements, varying in structural complexity, occur in other IncP plasmids (e.g. Tn4321 of R751; Partridge & Hall, 2003; Thorsted *et al.*, 1998) and indeed beyond the IncP family [e.g. the transposons in pWW53 (IncP-7); Yano *et al.*, 2007]. Their occurrence reinforces the importance of accessory genes in plasmid diversity and, via the potentially advantageous encoded properties, to niche-based adaptation of the bacterial host.

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