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Review

Transposase and cointegrase: specialized transposition proteins of the bacterial insertion sequence IS*21* and related elements

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Abstract. The bacterial insertion sequence IS*21* shares with many insertion sequences a two-step, reactive junction transposition pathway, for which a model is presented in this review: a reactive junction with abutted inverted repeats is first formed and subsequently integrated into the target DNA. The reactive junction occurs in IS*21*-IS*21* tandems and IS*21* minicircles. In addition, IS*21* shows a unique specialization of transposition functions. By alternative translation initiation, the transposase gene codes for two products: the transposase, capable of promoting both steps of the reactive junction pathway, and the cointegrase, which only promotes the integration of reactive junctions but with higher efficiency. This review also includes a survey of the IS*21* family and speculates on the possibility that other members present a similar transpositional specialization.

Key words. Transposase; cointegrase; insertion sequence; transposition; IS*21* family; IS*30*; IS*911*; retrovirus.

Discovery of the insertion sequence IS*21*

Transposition is defined as the translocation of a DNA fragment, the transposable element, from a donor site to a target site showing no sequence homology. Transpositional recombination events can promote different types of rearrangements such as the formation of deletions, inversions and replicon fusions [1]. When a conjugative plasmid undergoes replicon fusion with a bacterial chromosome, the chromosome can be mobilized from the origin of transfer provided by the plasmid, and conjugative chromosome transfer to recipient bacteria can occur. The ability of many conjugative plasmids to mobilize bacterial chromosomes is related to the activity of transposable elements [2]. The bacterial insertion sequence IS*21* was discovered during chromosome mobilization experiments in *Pseudomonas aeruginosa*. The broad-host-range plasmid R68.45, which promotes chromosome mobilization in *P. aeruginosa* and about 30 other bacterial species, owes this property to the presence of the tandemly repeated insertion sequence IS*21* [designated $(IS21)_2$]. The parental plasmid R68, which was first isolated from a clinical isolate of *P. aeruginosa*, carries a single copy of IS*21*. Spontaneous tandem duplication of IS*21* in R68 resulted in R68.45. Although R68.45 was obtained under laboratory conditions, there is evidence that $(IS21)_2$ can also form in nature [3–9]. R68.45 integrates into bacterial chromosomes at many different sites and thereby initiates chromosome transfer almost at random [2, 3, 9].

Transposition pathways of IS*21* and tandem formation

The transposition activities of IS*21* manifest themselves in different ways. A single copy of IS*21* can generate simple insertions, via a cut-and-paste (nonreplicative)

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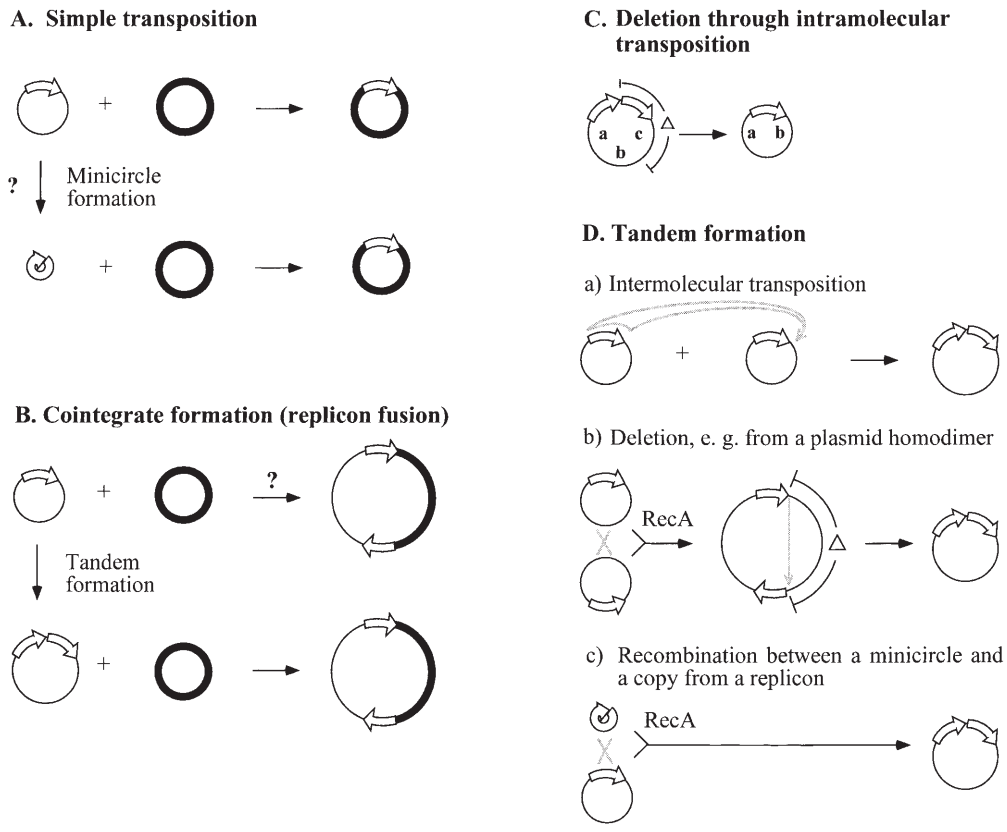


Figure 1. Overview of transposition pathways of IS*21*. Bold line, target replicon; thin line, donor replicon; Δ, deletion; arrows in grey represent site-specific recombination events; a cross stands for homologous recombination; figure modified from [39].

mechanism (fig. 1A) [10, 11]. This pathway is thought to involve circularization of IS*21* (fig. 1A, bottom part), an aspect that will be discussed in more detail at the end of this review. A single copy of IS*21* can also yield cointegrates at low frequency, probably through spontaneous tandem duplication of the element in the donor replicon before fusion with the target replicon; the resulting cointegrates show a copy of IS*21* in the same orientation at both junctions of the fused replicons (fig. 1B, upper part). By contrast, cointegrate formation (replicon fusion) between a replicon carrying $(IS21)_2$ and a target replicon is very efficient and also follows a cut-and-paste mechanism (fig. 1B, lower part) [10, 12]. The two or three nucleotides forming the IS*21*-IS*21* junction in the tandem are lost during the reaction [12, 13]. Both simple insertion and replicon fusion (fig. 1A, B) generate target duplications of 4 bp, rarely 5, 6 or 7 bp [13–15]. As in other transposition systems, the target duplications flanking the insert are formed by repair enzymes of the host [1, 16].

Although an $(IS21)_2$ structure is quite stable even in a $RecA^+$ host [8], deletions of one element can occur sometimes, and flanking DNA can be deleted concomitantly (fig. 1C), especially after conjugative transfer of an $(IS21)_2$ plasmid into a new host [8]. These deletion products are probably the consequences of intramolecular transposition of IS*21*.

Several models, which are not mutually exclusive, may explain IS*21* tandem formation. Simple intermolecular transposition of an IS*21* copy near one end of another IS*21* element is one explanation (fig. 1Da). Alternatively, an IS*21* element could transpose next to the copy situated on the other branch in a replication fork. Both mechanisms would involve site-specific recognition between two IS*21* termini [10, 17]. An IS*21* tandem can also result from a spontaneous deletion of a segment situated between two IS*21* copies present in direct orientation [10]. One way to generate a plasmid with two directly oriented IS elements is by RecA-dependent homodimerization (fig. 1Db). The deletion between two IS*21* elements would be a site-specific event as well. A further possibility leading to an $(IS21)_2$ structure would be a RecA-dependent recombination between an IS*21* copy carried by a replicon and an IS*21* minicircle (fig. 1Dc). At present, experimental evidence is available only for the deletion pathway (fig. 1Db) [10]. The other pathways (fig. 1Dac) have not been investigated because an appropriate selection system is lacking. In *P. aeruginosa*, the frequency of $(IS21)_2$ formation in R68 has been estimated to be $\leq 10^{-4}$ [2]. It emerges from the three proposed pathways of tan-

dem formation that in each case, at one time or another, two IS*21* ends come together in a site-specific recombination event.

Genetic organization of IS*21*

IS*21*, with 2131 bp, is among the largest insertion sequences. Its termini show inverted repeats (IRR and IRL) of 11 bp with one mismatch (fig. 2B, upper part). However, additional, less conserved sequence elements, termed multiple terminal repeats (MTRs), occur at both IS*21* termini (fig. 2AB) [15]. The consensus sequence for the MTRs of IS*21* is YRCCANYNNNRTNNNNCNNT, where Y and R represent pyrimidines and purines, respectively. Three MTRs are found at the left end, whereas two MTRs occur at the right end of IS*21* (fig. 2B, upper part). A potential integration host factor (IHF) recognition site is within MTR-L3 (fig. 2B, upper part).

When two IS*21* elements are organized in tandem, they are separated by a junction sequence of two or three variable nucleotides [11, 13]. The abutted IS*21* ends form a σ^{70} promoter whose −35 and −10 hexamers are located within IRR and MTR-L1, respectively (fig. 2B, upper part). This promoter drives the expression of the *istAB* genes in the downstream IS*21* element [13].

IS*21* contains two open reading frames (ORFs), the *istAB* genes, which are organized in an operon (fig. 2B). Both genes are necessary for efficient and accurate transposition activity [12, 13, 18]. They encode transposition and helper proteins, respectively, whose functions will be discussed below. As other transposable elements, IS*21* contains some additional short ORFs on both strands [19], but no product has been associated with them so far. The stop codon of *istA* and the start codon of *istB* overlap with one nucleotide in a stop-start configuration (fig. 2B), suggesting that expression of the *istA* and *istB* genes may involve translational coupling. By contrast, it seems highly unlikely that transposition of IS*21* depends on an IstAB fusion protein (produced by translational frameshifting), because separate expression of *istA* and *istB* does not affect the transpositional activity [11].

The function of the MTRs of IS*21* has not been assessed. However, in some other transposable elements such as bacteriophage Mu, Tn*7* and Tn*552*, MTRs are involved in the recognition of transposase and in the assembly of multimeric forms of this enzyme [20–25]. In the case of IS*21*, we note that the spacing of about 23 bp between the MTRs is such that the conserved sequence elements in the MTRs will be exposed to the same surface of the supercoiled DNA double helix [26, 27].

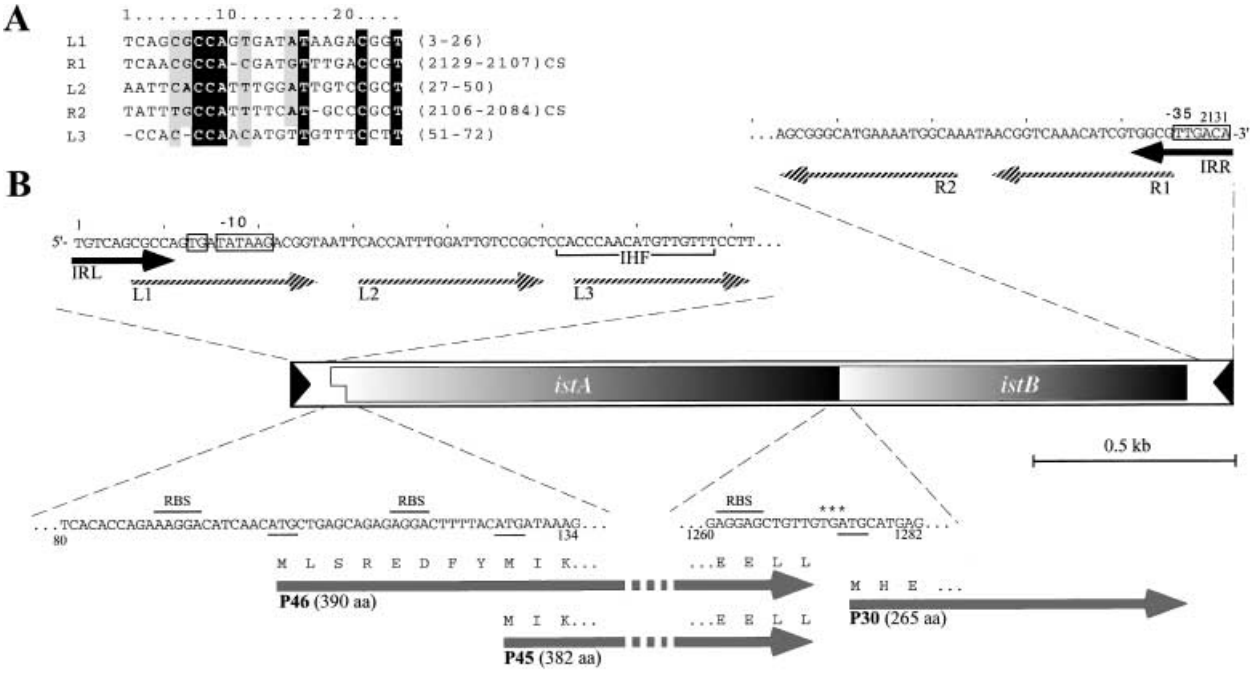


Figure 2. Insertion sequence IS*21*. (**A**) Alignment of the multiple terminal repeats (MTRs) together with their coordinates; CS, complementary strand; nucleotides outlined in black, identity; nucleotides outlined in grey, pyrimidine or purine; L3 is slightly less conserved and therefore not considered in the determination of the motif. (**B**) Upper part: termini of IS*21*; IRL and IRR, inverted repeats left and right; L1, L2, L3, R1 and R2, multiple terminal repeats; IHF, sequence resembling the consensus sequence for integration host factor binding; −10 and −35, sequences forming the promoter of the IR-IR junction. At the center: i*stA* and *istB*, the ORFs of IS*21*; black triangles represent the IRs. Lower part: products of the *istAB* genes; P46, the transposase coded by *istA*; P45, the cointegrase coded by *istA*; P30, the helper protein coded by *istB*; RBS, ribosome binding site; the start codons ATG are underlined; ***, stop codon.

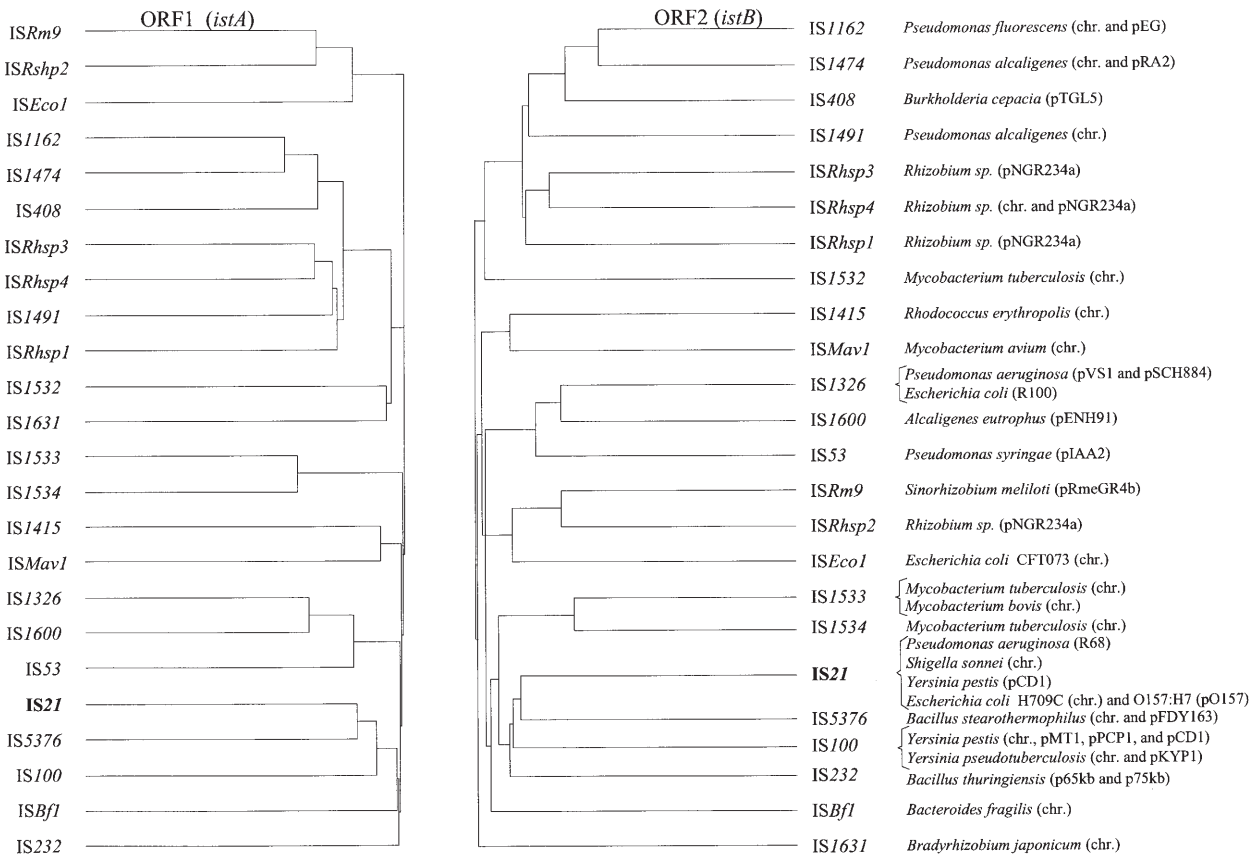


Figure 3. The IS*21* family. Dendrogram calculated by the Pileup program (GCG, University of Wisconsin, Madison) for both ORFs; the names of the bacteria refer to the organisms from which the elements have been isolated as a chromosomal copy (chr.) or a plasmid copy (name of the plasmid). The dendrograms obtained by Pileup represent the most similar pairs. The use of this approach is justified by the fact that the presence of divergent regions will not mask the close relatedness of the remainder of the sequences compared, especially of regions containing the active site.

When an IS*21*-IS*21* junction of a linearized plasmid was incubated with overexpressed IstA in a crude extract, about 30 nucleotides from both IS*21* ends were protected from nuclease attack [12], suggesting that MTR-L1 and MTR-R1 may interact with IstA during the replicon fusion reaction. If this interpretation is correct, then MTR-L1 might have an additional role in autoregulation of the *istAB* genes: IstA protein could block transcription by binding to MTR-L1 containing the –10 promoter sequence (fig. 2B).

A unique specialization of transposition functions in IS*21*: transposase and cointegrase

The *istA* gene is essential for both simple insertion and replicon fusion [11]. Two ATG start codons associated with ribosome binding sites (RBS) enable the *istA* gene to generate two products in the same reading frame, IstA(P46) and IstA(P45). These proteins of 46 kDa and 45 kDa, respectively, differ in their N-termini by eight amino acids (fig. 2B, lower part). Like transposases of several transposable elements, P46 and P45 show a helix-turn-helix motif at their N-termini (fig. 5A), probably promoting binding to DNA [13, 28]. The deduced amino acid sequences of IstA(P46) and IstA(P45) show a typical DDE motif (aspartate-122, aspartate-184, glutamate-230 in P46, cf. fig. 5A), which is part of the catalytic domain of retroviral integrases and numerous bacterial transposases [28, 29]. P46 and P45 have distinct functions. These were demonstrated with *istA* constructs that were engineered to express either IstA(P46) alone or IstA(P45) alone [11]. After overexpression in *Escherichia coli*, P45 promotes replicon fusion (cointegrate formation) at very high frequencies (10^{-1} to 10^{-2}) when the substrate is a reactive junction of two abutted IS*21* ends on a replicon and the target is a multicopy plasmid (e.g. fig. 1B, lower part) [11]. The optimal size of the IR-IR junction sequence is 2, 3 or 4 bp. With shorter or longer junction sequences (constructed artificially), the efficiency of replicon fusion decreases [11]. IS*21* ends joined via an artificial 4-bp (TATA) junction are slightly better substrates than those linked by a natural 2-bp (GT) or 3-bp (ACG) junction [11]. Because of this efficient enzymatic function in coin-

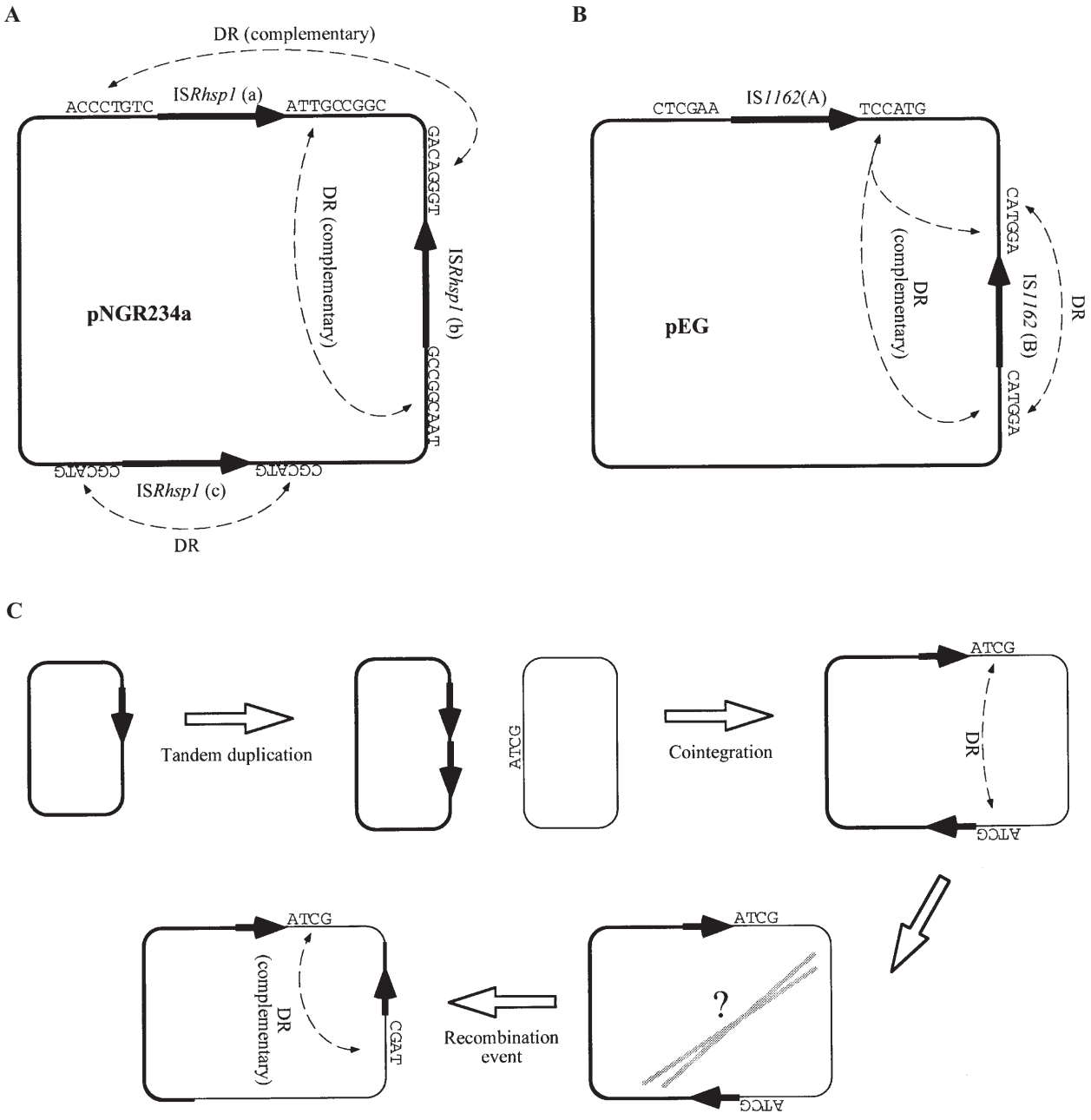
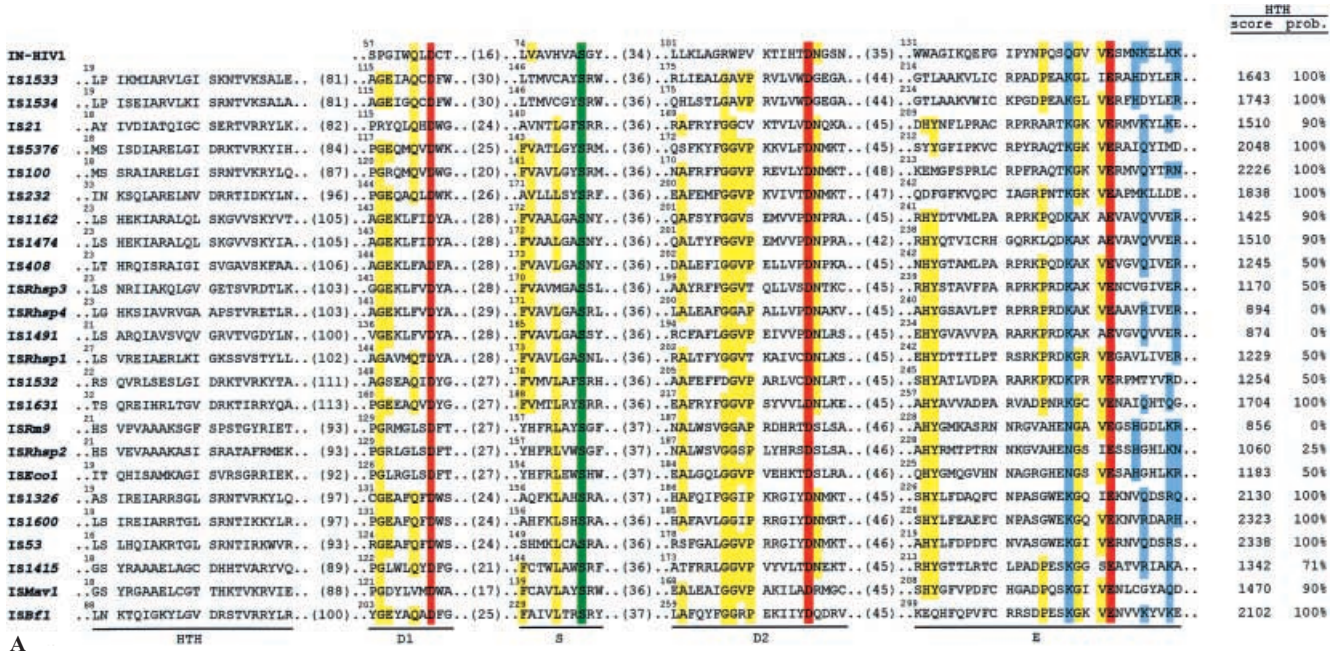


Figure 4. Cointegrate-like structures in the IS*21* family. (**A**) Localization and direct repeats of copies of IS*Rhsp1* on pNGR234a [66]. (**B**) Localization and direct repeats of copies of IS*1162* on pEG [58]. (**C**) Proposed molecular mechanisms to explain the complementary direct repeats. The insertion sequences are represented by arrows (leading from IRL to IRR); the sequences of the direct repeats (DR) are shown on the same strand; in the proposed model the tetranucleotide of the target site shown in the proposed model is arbitrary; ?, recombination event inverting a DNA segment.

tegrate formation, P45 has been named cointegrase. By contrast, when the substrate is composed of two distant IS*21* ends as in a single IS*21* element on a replicon, cointegrase has poor activity: simple insertions (fig. 1A) are formed at $\leq 10^{-6}$ by this enzyme [11]. P46 displays an equal and intermediate activity (ca. 10^{-3}) in both the simple transposition and the replicon fusion pathways (figs. 1A and 1B, lower part) [11]. For this reason, P46 has been designated transposase.

Mechanistically, the cleavage and strand transfer reactions catalyzed by cointegrase and transposase are probably very similar; we will discuss them at the end of this review. What then might be the role of the extra eight amino acids (MLSERDFY) at the N-terminus of trans-



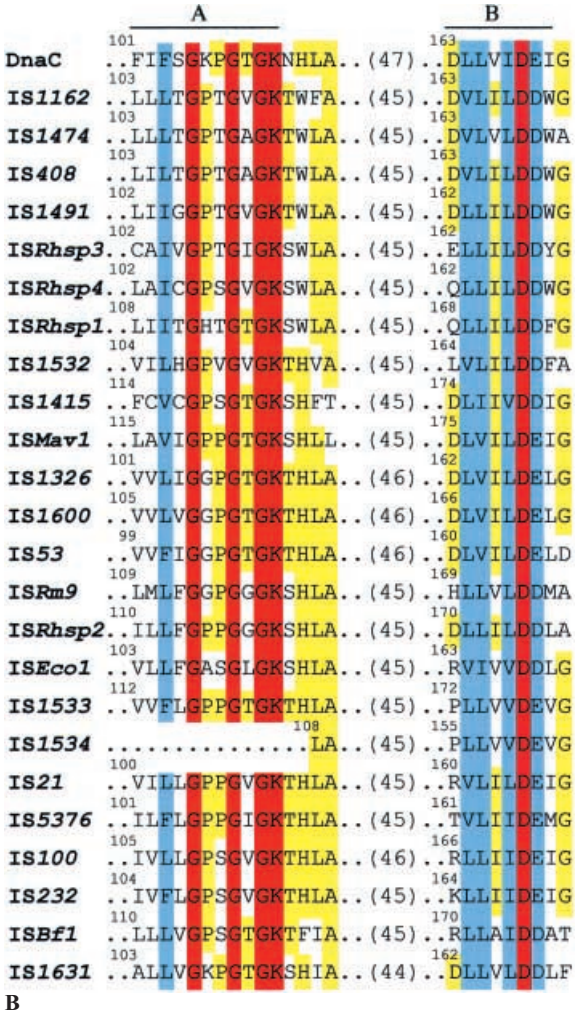


Figure 5. Alignments of the ORFs of the IS*21* family. (**A**) The amino acids of the DDE region of the ORF1 (*istA*) of the IS*21* family members and of IN-HIV1 [87]; HTH, helix-turn-helix motif; D1, D2 and D3, regions including the DDE triad; S, region including a well-conserved serine (see text); on the right, the score of the HTH motif calculated according to [86] and the probability of existence are shown; even if the scores obtained for IS*Rsp4* and IS*1491* predict no HTH motif, the analysis of these sequences by the program hthscan (GCG, University of Wisconsin, Madison) identifies them as probable HTH; the numbers in brackets represent the number of residues between the HTH motif and the first D, the first D and the S, the S and the second D, the second D and the E, respectively; the numbers above the sequences are the coordinates; yellow, residues conserved in the majority of cases; red, residues strictly conserved belonging to the DDE triad; green, serine strictly conserved; blue, basic residues neighboring the E. (**B**) Motifs of the ORF2 (*istB*) of the IS*21* family members and of DnaC from *E. coli* [32]; the numbers above the sequences are the coordinates; in brackets are the number of residues not represented; red, strictly conserved residues; blue, conserved similar residues; yellow, residues conserved in the majority of cases; the A domain of IS*1534* is deleted.

posase? These residues may help the transposase to bring two IS*21* ends close to each other, such that they can form an IR-IR junction which is subsequently inserted into the target DNA. The N-terminal residues of P46 and P45 might also influence the accessibility of the putative transposition donor complex to the target DNA.

Both the simple transposition (fig. 1A) and the replicon fusion (fig. 1B) pathways need a helper protein, IstB, encoded by IS*21*. This 30-kDa protein appears to have a function similar to that of MuB of bacteriophage Mu and TnsC of transposon Tn*7* [30, 31]. IstB has an ATP/GTP binding motif which is also found in DnaC [32] (cf. fig. 5B). The absence of IstB during replicon fusion drastically reduces the efficiency of the reaction and generates atypical target duplications and/or deletions in the target. Thus, IstB is needed for accurate strand transfer and capture of the target DNA [18]. Under optimal conditions the cointegration reaction proceeds at a frequency of 10^{-1} without being lethal to the *E. coli* host [11]. It is likely that this frequency represents an initial burst of activity and that a mechanism exists which prevents further insertions of IS*21* into the same target DNA, i.e. an immunity mechanism exists. Circumstantial evidence for immunity against multiple IS*21* insertion has been reported [17] and confirmed in our laboratory, but the mechanism involved has not been elucidated. By analogy with MuB of phage Mu and TnsC of Tn*7*, IstB might have a role in establishing immunity [23, 33–36].

Target selection of IS*21* in vivo and in vitro

The high replicon fusion activity of cointegrase, assisted by IstB, has been exploited to generate an in vitro system in which a reactive IS*21*-IS*21* junction is carried by a suicide plasmid [18]. Crude *E. coli* cell extracts containing overproduced cointegrase and IstB catalyze replicon fusion between the suicide construct and a multicopy target replicon at a frequency of ca. 10^{-3} (per target replicon) [15, 18].

The target specificity of IS*21* has been investigated by an analysis of replicon fusion products obtained in vivo and in vitro with IstA(P45) and IstB. More than 100 insertion sites have been sequenced [14, 15]. No consensus target sequence has become apparent, in contrast to an earlier proposal based on a small number of insertion sites [37]. However, the IS*21* transposition machinery appears to prefer targets displaying an 11-bp periodicity of the dinucleotide AA flanking the insertion sites. This sequence organization, which may confer anisotropic flexibility to the target DNA, suggests that insertions at the center of S-shaped DNA would be favored [B. Berger, T. Junier, D. Haas and A. Stasiak, unpublished results]. A more pronounced target preference showing an intrinsic S-shape has been revealed for IS*231A*, an element belonging to the IS*3* family [38].

A survey of the IS*21* family

IS*21* is the prototype of a growing family of bacterial insertion sequences. Since publication of previous reviews [39, 40], several new members have been added to the IS*21* family. Our first objective here is to describe the current status of this family.

Members of the IS*21* family are characterized by two *istA*- and *istB*-like ORFs, by terminal IRs of usually up to 50 bp which are not conserved within the family but generally end with CA, by the presence of MTRs of 15–20 bp at both ends, and by target duplications (direct repeats) of 4–9 nucleotides. These elements are listed in table 1. Elements whose amino acid sequences of the ORFs diverge by less than 5% are defined as isoforms. When dissimilarity is obviously the result of translation frameshifts (due to genuine mutations or possibly due to sequencing errors), nucleotide sequences showing less

Table 1. Origins of the insertion sequences of the IS*21* family.

IS element[a]	location[b]	Organism	Reference/ accession no.[c]	DR[d] (bp)	Comments on transpositione[e]
IS*21*	R68	*Pseudomonas aeruginosa*	[13] X14793	4 (5, 6, 7)	simple transposition,
	pO157	*E. coli* O157:H7	[41] AF74613 (T)		tandem formation,
	chr	*E. coli* H709C	[42]		cointegrate formation
	pB171	*E. coli* B171	[43] AB024946 (T)		(see text)
	chr.	*Salmonella enterica*	AJ242964 (T)		
	chr.	*Salmonella typhi*	AB029403 (T)		
	RK2	diverse	[44]		
	RP1				
(IS*8*)	RP4				
(IS*640*)	pCD1	*Yersinia pestis*	[45] AF053946 (T)		
(IS*21p*)	pCD1	*Yersinia pestis*	[46] AF074612 (T)		
(IS*640*)	chr.	*Shigella sonnei*	[47] X05956 (P)		
IS*53*	pIAA2	*Pseudomonas syringae*	[48] M83932	8	–

Table 1 (continued)

IS element[a]	Location[b]	Organism	Reference/ accession no.[c]	DR[d] (bp)	Comments on transposition[e]
IS*100*	chr.	*Yersinia pestis*	[49] AL031866	5	transposition [53]
	pMT1	*Yersinia pestis*	[45] AF053947 [50] AF074611		
	pPCP1	*Yersinia pestis*	[51] Z32853 [45] AF053945		
	pCD1	*Yersinia pestis*	[52] X78302 [45] AF053946 [46] AF074612		
	?	*Yersinia pestis*	L19030		
	chr.	*Yersinia pseudotuberculosis*	AJ236887		
	pKYP1	*Yersinia pseudotuberculosis*	[54] U59875		
	pB171	*E. coli* B171	[43] AB024946 (T)		
IS*232*	p65kb	*Bacillus thuringiensis*	[55] M38370	8, 6	simple transposition [55]
	p75kb	*Bacillus thuringiensis*	M77344		
IS*408*	pTGL5	*Burkholderia cepacia*	[56] L09108	8	transposition [57]
IS*1162*	pEG	*Pseudomonas fluorescens*	[58] X79443	6, 7	simple transposition [58]
	chr.	*Pseudomonas fluorescens*	[58]		replicon fusion without duplication of the element [58] cointegrate-like structure observed (see text)
IS*1326*	pVS1	*Pseudomonas aeruginosa*	[59] U38187	ND	–
	pSCH884	*Pseudomonas aeruginosa*	[59]		
	R100	*Escherichia coli*	AP000342		
IS*1415*	chr.	*Rhodococcus erythropolis*	[60] AF002247	5, 6	simple transposition [60]
IS1*474*	chr.	*Pseudomonas alcaligenes*	[61] U67315	4	replicon fusion, cointegrate formation with IS*1475*? [61]
IS*1475*	chr.	*Pseudomonas alcaligenes*	[61] U67315 (T)	ND	replicon fusion, cointegrate formation with IS*1474*? [61]
IS*1491*	chr.	*Pseudomonas alcaligenes*	[62] U84154	ND	simple transposition [62] replicon fusion, cointegrate formation? [62]
IS*1532*	chr.	*Mycobacterium tuberculosis*	[63] Z95389 to Z77165	ND	–
IS*1533*	chr.	*Mycobacterium tuberculosis*	[63] Z83858	5	–
	chr.	*Mycobacterium bovis*	[64] AJ238712		
IS*1534*	chr.	*Mycobacterium tuberculosis*	[63] Z95436	5	defective by deletion in ORF2?
IS*1546*	pRE4	*Pseudomonas putida*	[65]	ND	–
IS1600	pENH91	*Alcaligenes eutrophus*	[66, 67] D64144	ND	cointegrate-like structure observed, except for the DR [66]
IS*1631*	chr.	*Bradyrhizobium japonicum*	[68] AB011*021* (P)	ND	–
IS*5376*	pFDY163	*Bacillus stearothermophilus*	[69] X67861	5	transposition [69, 70]
IS*Atu1*	pTiA6NV	*Agrobacterium tumefaciens*	AF034854 (T)	ND	–
IS*Bf1*	chr.	*Bacteroides fragilis*	[71] U05888	ND	–
IS*Bf2*	chr.	*Bacteroides fragilis*	[71] U05886 (P)	ND	–
IS*Che1*	chr.	*Chelatobacter heintzii*	[72] L49438 (P)	ND	–
IS*Cpi1*	chr.	*Carnobacterium piscicola*	[73] L471*21* (T)	ND	–
IS*Eco1*	chr.	*Escherichia coli* CFT073	[74] AF081285	5	–
IS*Lla1*	chr.	*Lactococcus lactis*	AF064765 (P)	ND	–
IS*Mav1*	chr.	*Mycobacterium avium*	[75] AF125999 A63806	5	–
IS*Pae1*	chr.	*Pseudomonas aeruginosa*	AF087482 (T)	ND	–
IS*Psesp1*	chr.	*Pseudomonas* sp.	[67] AB019033 (T)	ND	–
IS*Rm9*	pRmeGR4b	*Sinorhizobium meliloti*	[76] Y13432	7	–

Table 1 (continued)

IS element[a]	Location[b]	Organism	Reference/ accession no.[c]	DR[d] (bp)	Comments on transposition[e]
IS*Rhsp1*	pNGR234a	*Rhizobium* sp.	[77] AE000081 AE000066 AE000097	6, 8, 9 (see text)	cointegrate-like structure observed (see text)
IS*Rhsp2*	pNGR234a	*Rhizobium* sp.	[77] AE000079 AE000086 AE000095	6, 7	–
IS*Rhsp3*	pNGR234a	*Rhizobium* sp.	[77] AE000099	8	–
IS*Rhsp4*	chr. pNGR234a	*Rhizobium* sp. *Rhizobium* sp.	[77] AE000090 [78]	7, 8, 9	defective by insertion of other IS elements?
IS*Sco1*	chr.	*Streptomyces coelicolor*	AL023861 (T?)	ND	–

[a] Elements shown in brackets are isoforms. IS*8* and IS*21* are probably identical.
[b] Location indicates plasmids or chromosomes (chr.).
[c] (P), partial DNA sequence; (T), truncated copy.
[d] DR, direct repeat = target duplication; ND, not determined.
[e] Experimental evidence established for the transpositional movements indicated.

than 10% divergence are considered as isoforms. Some of the elements of table 1 are truncated, incompletely sequenced or defective.

As the transposition activity of many members of the IS*21* family has not been tested (table 1), it is quite difficult to distinguish between sequencing errors and real mutations which could affect transposition activity. We are aware that the sequence corrections introduced by us (indicated by 'M' in table 2) favor the phylogeny to function.

In the elements listed in table 2, ORF1 (340–585 amino acid residues) is always longer than ORF2 (245–270 amino acid residues). The ORFs are frequently in an overlapping stop-start or start-stop configuration, arrangements that may allow translational coupling. In a few elements, even a superimposition of up to 115 bp has been reported. In no case does there seem to be a motif involved in a programmed frameshift between ORF1 and ORF2.

At both ends of the elements there are imperfect inverted repeats, which include two to seven variably conserved MTRs of about 20 bp each, with a spacing of generally 23–24 bp and occasionally 33–34 or 46–48 bp, i.e. about multiples of 11.3 bp, the supercoiled DNA double helix turn [26, 27]. The members of the IS*21* family show a CA dinucleotide or exceptionally a TA dinucleotide at the termini. The terminal CA exists in many bacterial elements which code for transposases carrying a DDE motif, and also at the termini of the integrated forms of retroviruses [28, 29]. In the case of IS*21*, a CA to GA transversion affects cointegrate formation by a factor of about 50, and a CA to CT transversion reduces activity to a nonmeasurable level [79]. Thus, the two ends observed in the IS*21* family differ from one another only in the penultimate, less important nucleotide. The dinucleotides CA and TA are both 'kinkable' [80], and this might facilitate cleavage at their 3′ end by integrase, transposase or cointegrase.

The IS*21*-IS*21* junction of R68.45 forms a strong promoter whose activity was demonstrated by an *istA-galK* fusion in the downstream element [13]. Such promoter sequences, discovered in other IS families as well [81], are expected for other members of the IS*21* family, e.g. for IS*1415* and IS*5376*. Potential promoters can also be identified upstream of ORF1, e.g. in IS*1326* and IS*53*, but they are less canonical. Whether such promoters exist in reality is uncertain because for no member of the IS*21* family, except for IS*21* itself, has a reactive IR-IR junction been described.

An alignment of ORF1 and ORF2 proteins of the IS*21* family was performed using the Pileup program (fig. 3). For both ORFs, similar subgroups of related elements appear, for instance IS*21* with IS*5376* and IS*100*, or IS*1326* with IS*1600* and IS*53*. The proximity of IS*21* (from various Gram-negative bacteria) and IS*5376* (from a Gram-positive bacterium) in the dendrogram suggests their mobility, probably via plasmid transfer. Another similar example is provided by the closely related elements IS*1532* (found in *Mycobacterium tuberculosis*) and IS*1631* (found in *Bradyrhizobium japonicum*) (fig. 3). On the other hand, IS elements from the same organism (e.g. IS*1532* and IS*1533* from *M. tuberculosis*) need not be closely related to each other.

Data on the transposition activities of IS*21* family members are scarce (see references in table 1). Apart from IS*21*, only IS*1162* has been shown to give simple insertion and replicon fusion, the latter without apparent duplication of the element. Simple insertion of some elements, e.g. IS*232* and IS*1491*, and sometimes replicon fusion, e.g. of IS*1474* and IS*1491*, have been observed, but repli-

Table 2. Properties of the elements of the IS*21* family.

Element	DNA [bp]	ORF1 [aa][a]	ORF2 [aa][a]	Alternative start in ORF1[b]	Intergenic ORF1-ORF2 structure[c]	Multiple terminal repeats[d]		Termini[d]	
						Left	Right	Left	Right
IS*21*	2131	390	265	ATG/ATG (8)	Stop-Start TGATG	1 2 3	1 2	CA	CA
IS*53*	2555	496 M	267 M	ATG/ATG (9)	superposition 8 bp	1 2	1 2	CA	CA
IS*100*	1954	340	259	ATG/ATG (7)	Stop-Start TGATG	1 2 3	1 2	CA	CA
IS*232*	2183	431	250	absence	superposition 8 bp	1 2 3	1 2	TA	CA
IS*408*	>2530	518	>231	ATG/ATG (7)	spacing 16 bp	1 2 3	1 2	CA	CA
IS*1162*	2634	558	249 M	ATG/ATG (7)	superposition 115 bp	1 2 3 4 5 6	12	CA	CA
IS*1326*	2470	507	260	GTG/GTG (3)	superposition 11 bp	1 2 3	1 2	CA	CA
IS*1415*	2580	513	263	ATG/ATG (7)	Stop-Start TGATG	1 2 3 4	1 2 3 4	CA	CA
IS*1474*	2633	558	249	ATG/ATG (7)	superposition 113 bp	1 2 3 4 5 6 7	1 2	CA	CA
IS*1491*	>2489	509	251	GTG/ATG (5)	spacing 17 bp	present, but the sequence of the element is apparently partial		?	?
IS*1532*	2645	511 M	251	ATG/ATG (7)	spacing 97 bp	1 2 3	1 2	CA	CA
IS*1533*	2213	413	266 M	absence	Stop-Start TGATG	1 2 3	1 2 3	CA	CA
IS*1534*	2130	411 M	248	absence	Stop-Start TGATG	1 2	1 2	CA	CA
IS*1600*	2520	518	264	GTG/GTG (3)	superposition 11 bp	1 2	1 2	CA	CA
IS*1631*	>2712	585	255	ATG/GTG (11)	spacing 4 bp	present, but the sequence of the element is apparently partial		?	?
IS*5376*	2107	400	251	ATG/ATG (8)	Start-Stop ATGA	1 2	1 2	CA	CA
IS*Bf1*	2787	582	263	long N-terminal extension	superposition 32 bp	?	?	CA	CA
IS*Eco1*	2407	499 M	250	absence	Start-Stop GTGA	1 2	1 2	TA	TA
IS*Mav1*	2536	500 M	264	ATG/ATG (7)	Start-Stop ATGA	1 2 3	1 2	CA	CA
IS*Rm9*	2798	502	270	absence	Start-Stop ATGA	1 2 3 4	1 2 3	CA	TA
IS*Rhsp1*	2623	516	263	ATG/ATG (11)	Start-Stop GTGA	1 2 3 4	1 2 3 4	CA	CA
IS*Rhsp2*	2641	504	298	absence	Start-Stop ATGA	1 2	1 2 3	CA	TA
IS*Rhsp3*	2786	514	248	TTG/ATG (7)	spacing 11 bp	1 2 3 4 5	1 2 3	CA	CA
IS*Rhsp4*	2599	516 M	245	ATG/ATG (7)	spacing 11 bp	1 2 3 4	1 2 3	CA	CA

[a] Where modifications of the nucleotide sequence were introduced, "M" is added under the size of the ORF; aa, amino acid residues.
[b] Two start codons are given with the size difference (in amino acids residues) between the putative transposases and cointegrases.
[c] Description of the region including the stop of ORF1 and the start of ORF2.
[d] The numbers of the multiple terminal repeats are given only when the sequence of the IS termini is complete; ?, not determined.

con fusion products have not been examined. The proximity of some copies of IS*100* in *Yersinia pestis* or IS*1600* in *Alcaligenes eutrophus* suggests transposition of composite elements or cointegration, but the direct repeats normally generated by such mechanisms are not apparent. By contrast, the arrangement of IS*Rhsp1* and IS*1162* copies on plasmids pNGR234a and pEG, respectively, could be due to a cointegration mechanism. However, in both cases, the transposition event producing the cointegrates is probably followed by a recombination event inverting a segment carrying one element (fig. 4). These examples illustrate that simple transpositions and replicon fusions can occur among IS*21* family members, but these processes have mostly been inferred, not directly observed.

Does a cointegrase/transposase specialization exist in other members of the IS*21* family?

Here we analyze the genetic organization of the 5′ end of ORF1 in various elements, using IS*21* as a model. The *istA* gene of IS*21* uses two alternative start sites located 24 bp apart to produce transposase and its truncated form, cointegrase (fig. 2B) [11]. The difficulty in determining in silico whether alternative start sites exist for ORF1 arises from the fact that it is difficult to predict the effectiveness of potential ribosome binding sites and, moreover, in different organisms [82–84]. Furthermore, alternative start codons (GUG or UUG instead of AUG) may be used with various degrees of preference in different bacterial species: in *E. coli* AUG (83%) > GUG (14%) > UUG (3%); in *B. subtilis* AUG (78%) > UUG (13%) > GUG (9%) and in *M. tuberculosis* AUG (61%) > GUG (33%) >UUG (5%) [85].

In the absence of stringent criteria, inspection of ORF1 sequences suggests that most members of the IS*21* family probably use two alternative translation start sites, spaced 3 to 11 codons apart (table 2). The elements which are devoid of potential alternative sites of translation initiation for ORF1 represent a minority; two of them occur in *Mycobacterium spp.* (IS*1533* and IS*1534*) and four of them show at least one TA terminus (IS*232*, IS*Eco1*, IS*Rm9* and IS*Rhsp2*). The eight amino acid residues truncated in IS*21* cointegrase are mostly polar (fig. 2B). A similar preference is also found in ORF1 of IS*53*, IS*1415*, IS*1491*, IS*1631*, IS*Mav1* and IS*Rhsp1*, but other elements do not conform to this rule. Thus, the polarity observed at the N-terminus of IS*21* transposase could be fortuitous.

Domains of ORF1 and ORF2 proteins in the IS*21* family

For both *istA* products of IS*21* and for all ORF1 products of the members of the IS*21* family (except IS*Rm9*), a helix-turn-helix motif can be deduced in the N-terminal part (fig. 5A). Such a motif is predicted to provide DNA binding activity [86], which is consistent with the observation that numerous bacterial transposases have a DNA binding domain in their N-terminal part [28]. In the C-terminal part, few residues are strictly conserved among all elements of the family (a global alignment not shown, but see [39]).

The catalytic domain highlighted by the DDE triad, which is present in numerous bacterial transposases and retroviral integrases [28, 29, 87], is conserved in ORF1 of the IS*21* family (fig. 5A). A serine residue, which is situated halfway between the two aspartates in retroviral integrases (IN) such as IN HIV-1, is also conserved in the IS*21* family (fig. 5A). Thus, four regions can be defined (fig. 5A), one (S) including the conserved serine residue and the three others (D1, D2 and E) including the DDE triad and approximately corresponding to the regions referred to as N2, N3 and C1 by other authors [29, 88].

The importance of the DDE residues has been confirmed by directed mutagenesis of several retroviral integrases (for a review see [89]) and of transposases from Mu [90], IS*10* [91], IS*911* [29] and Tn*7* [92]. In the case of IN, mutations of D64, D116 or E152 abolish processing and strand transfer as well as disintegration, an enzymatic activity whose biological significance is not well defined and which dissociates integrated viral DNA from the target. In bacterial transposases, donor cleavage (equivalent to processing) and strand transfer are equally affected by mutations in the DDE triad, which is the catalytic core domain of transposases and integrases through coordination of essential divalent metal ions (especially Mg^{2+}) [29].

Although the conserved serine probably is not part of the catalytic site, S81A or S81G mutations in IN seriously impair the activity of the enzyme, probably by affecting folding [93, 94]. Structural analyses of IN of HIV-1 and MuA transposase show that this serine and its threonine homolog in MuA form part of a junction between two β sheets [95, 96]. However, it has been shown for Tn*7* that the analogous S304A mutation in TnsB (forming a heterodimeric transposase with TnsA) has no effect on transposition [92, 97].

Other residues of the regions D1, D2 and E are relatively well conserved in the IS*21* family (fig. 5A), and one can find hypothetical functions based on studies of IN. The Q62 residue of IN HIV-1, which interacts with the penultimate nucleotide C at the viral 5′ end [98], is present in about half of the elements of the family (fig. 5A). Residue Q148 of IN HIV-1, which also recognizes the 5′ end [99], has a lysine counterpart in most elements of the family (fig. 5A). Downstream from E152 of IN HIV-1 and located on the same side of the α helix [100], the residues K156 and K159 are involved in the positioning of the final dinucleotide CA [101]. Although these lysine residues are present in a few elements of the family (including IS*21* itself), other basic or polar residues, at identical or close positions, might play the same role. In addition, E152 appears to be involved in the specific recognition of the CA end by IN HIV-1 [99]. In the IS*21* family, the spacing between the first aspartate of the DDE triad and the conserved serine is variable, unlike the spacing separating the conserved serine from the second aspartate, which varies between 36 and 37 residues (fig. 5A). Whereas a number of other transposases and integrases have a DD(35)E arrangement, spacing between the second aspartate and glutamate is larger in IS*21* and related elements, extending from 42 to 48 residues (fig. 5A). It is possible that this spacing could form a loop playing a role in the control of the active site, as proposed for MuA where the motif is DD(55)E [96].

The ORF2 proteins of the IS*21* family, of which the IstB protein from IS*21* is the prototype, are better conserved than ORF1 proteins (fig. 5B; a total alignment of some elements is given in [39]). ORF2 proteins all have an ATP/GTP binding motif, similar to the motifs of the DnaA and DnaC proteins [32]. Other proteins of transposable elements showing the same type of motif are MuB of bacteriophage Mu [31, 102, 103] and TnsC of Tn*7* [104], for which ATP binding has been demonstrated, TnpB of Tn*552* [25, 105], and TniB of Tn*402* (=Tn*5090*) [106] and Tn*5053* [107, 108]. Like DnaC, these proteins are likely to form part of 'molecular matchmakers', which would use ATP for transient conformational changes promoting protein-DNA interactions [109]. These proteins can also be seen as ATP-dependent switches that control the assembly and activation of the transpososomes and may play a role in transposition immunity [35, 110].

Mechanistic aspects of the transposase/cointegrase specialization in the reactive junction pathway

Recent work on several bacterial insertion sequences has provided evidence for an intermediate, the IS minicircle, where the two ends of the transposable element are joined by a few base pairs [40]. IS minicircles and tandems share an important feature, i.e. an IR-IR junction. For IS*1*, a model including minicircle and tandem formation was proposed 20 years ago [111].

Several insertion sequences have been shown to form tandem and minicircle intermediates; tandem structures of IS*30*, IS*21*, IS*3*, IS*2* and IS*911* have been described [10, 13, 16, 112–115], whereas minicircles have been found for IS*911*, IS*21*, IS*3*, IS*1*, IS*2* and IS*30* [116–124]. These structures are both formed via an interaction between two separated IS ends, and the subsequent transpositional integration of these intermediates follows a similar pathway. These considerations lead us to propose a unifying model (fig. 6), which is based on findings reported for IS*911* and, to some extent, for IS*21* and IS*30*. We call this the reactive junction pathway. Similar models have previously been presented by others, especially by the groups of W. Arber, M. Chandler and F. Olasz [16, 29, 81, 113, 124].

IS*911* is a member of the IS*3* family [40]. The formation of IS*911* minicircles (fig. 6, 1st step) requires the OrfAB product, the transposase assembled by a programmed –1 frameshift occurring between two consecutive and partially superimposed ORFs [125], and probably some host functions to resolve the 'figure-eight' molecule, which results from the transfer of a single strand at one end of the IS element to the same strand close to the opposite IS terminus [117, 126]. Although linear forms of IS*911* originating from minicircles can give simple insertions in vitro [127], the main transposition pathway of IS*911* seems to proceed directly via minicircles [81, 128]. These are cleaved by the OrfAB product at the reactive IR-IR junction by two consecutive or simultaneous single strand cuts at the 3′ ends, which are then transferred to the target DNA (fig. 6, 2nd step). The integration of the minicircles is highly stimulated by the simultaneous presence of the OrfA product, which contains the helix-turn-helix motif but lacks the DD(35)E catalytic domain of OrfAB [81, 128]. Both OrfA and OrfAB contain a leucine zipper motif, which allows OrfAB oligomerization [129] and the regulation of IS*911* transposition through interaction of OrfA with OrfAB within a transposition complex [130]. Target capture appears to be an important function of OrfA [130]. Recently, tandem formation by site-specific recombination between the ends of two IS*911* copies on the same dimeric plasmid molecule has been shown to occur (as shown in fig. 1Db) and to produce the same type of IR-IR reactive junction as in minicircles [16]. In summary, the OrfAB product is not only necessary for the formation of minicircles but also for their integration into the target DNA.

IS*30* is a bacterial insertion sequence for which tandem formation was reported earlier [112]. Members of the IS*30* family have one ORF [40]. This codes for a transposase whose N-terminal part interacts specifically with the IRs of the element [131] and whose C-terminal part comprises a DDE motif. The highly reactive and unstable structure $(IS30)_2$ promotes replicon fusion (cointegration) and many rearrangements, including loss of one of the elements from the dimer (dimer dissolution) and deletion of adjacent DNA [113, 124]. The formation of IS*30* minicircles as intermediates in the simple transposition pathway was recently demonstrated [124]. IS*30* shows a duality in its specificity of insertion, with a preference for targets adjacent to IRs [132] and a not very rigorous hotspot [37]. Mutant forms of IS*30* transposase with deletion of 31 or 81 amino acid residues at the C-terminus are defective for tandem formation, dimer dissolution and replicon fusion, but retain some transpositional activity with a preformed IS*30*-IS*30* junction as a substrate [133], illustrating the fact that the presence of an IR-IR junction can simplify the catalytic task of the transposase by bypassing the 1st step (fig. 6).

IS*21* tandems have been studied extensively, as they have high cointegration activity [13]. For unknown reasons, $(IS21)_2$ is more stable than tandems of other IS elements. This fact has facilitated studies on $(IS21)_2$-mediated cointegrate formation. Interestingly, the spacing between abutted IS elements in tandems and minicircles corresponds to the target duplication for IS*911* (3 bp) and IS*30* (2 bp), but this is not the case of the spacing observed in $(IS21)_2$ (2 or 3 bp instead of the standard 4-bp target duplication) [11]. It was realized early that a plasmid carrying $(IS21)_2$ could be considered as a circular transposable

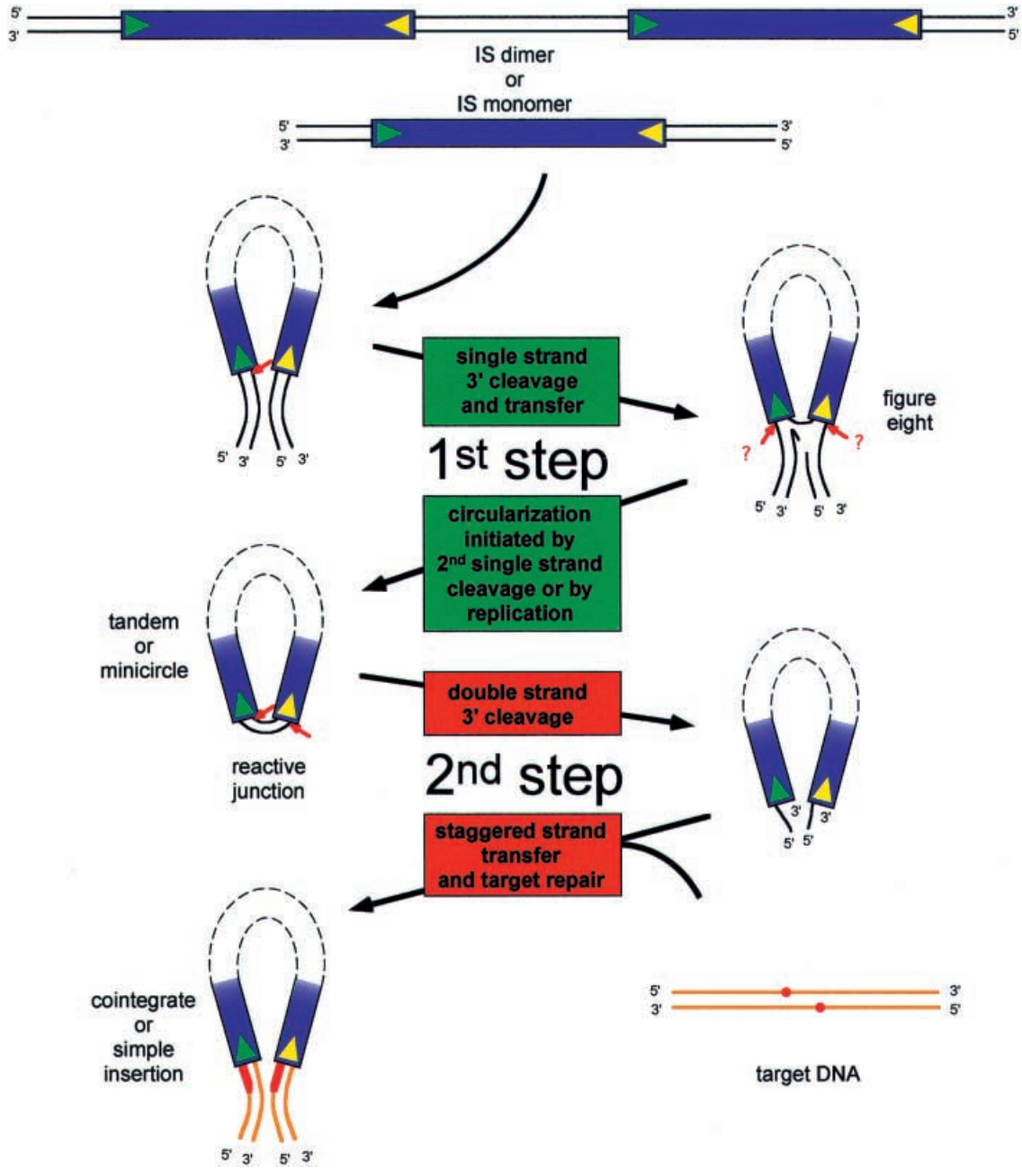


Figure 6. General model for the reactive junction transposition pathway. IS elements are shown in blue with yellow and green inverted repeats, backbone donor DNA in black, target DNA in orange, target duplications resulting from DNA repair of the target in red; red arrows show the single strand 3′ cleavages; red dots on the target DNA illustrate the staggered cuts.

element [10]. Such a structure has features similar to those of the circular intermediate structures (minicircles) postulated for other elements. Plasmids containing a single copy of IS*21* appear to release minicircular IS*21* in *E. coli* at about 10^{-3} with only the IstA function being required [118]. Although the structures of the putative IS*21* minicircles have not been entirely elucidated, we presume that they contain an IR-IR junction of the type found in $(IS21)_2$. This speculation is supported by analogy with tandems and minicircles of IS*911* and IS*30* [16, 124].

Transposase is 10,000 times more effective than cointegrase at promoting the simple transposition pathway of IS*21* [11]. This implies that transposase can bring together two ends of an IS*21* copy, e. g. by forming a figure eight (fig. 6, 1st step), whereas cointegrase cannot. The difference resides in the eight N-terminal amino acids of transposase, but remains unexplained mechanistically. Since the formation of an IR-IR junction in a tandem similarly requires the synapsis of two IS*21* ends – irrespective of the precise mechanism (fig. 1D) – we presume that transposase catalyzes this step.

Integration of an IR-IR junction into a target proceeds optimally in the presence of cointegrase and IstB [11, 18], and integration of natural minicircles and tandems can be expected to follow the same pattern (fig. 6, 2nd step). With respect to the insertion sequences discussed above, IS*30* transposase artificially truncated at the C-terminus retains cointegrase function and OrfA of IS*911*, like IstB, helps capture and integration into the target.

Integration of an IR-IR junction can also occur in the presence of transposase and IstB, albeit 100 times less efficiently than with cointegrase [11]. Therefore, the specialized cointegrase function can be seen as a means of accelerating the second step of the nonreplicative transposition pathway shown in figure 6, i.e. the reaction of two abutted IS ends with a target.

Concluding remarks

In nonreplicative transposition of insertion sequences and transposons, both strands of the transposable element are freed from the donor backbone at both ends of the element. This process can involve a hairpin intermediate with sequential cleavages of both strands as in Tn5 and Tn*10* transposition [134, 135], or the formation of structures with abutted IRs creating a reactive junction, as discussed here and in [136]. The reactive IS*21*-IS*21* tandem, once perceived as a curiosity in the world of insertion sequences [6, 10], now finds a place in the more general reactive junction pathway, which may be used not only by IS*21*, IS*911* and IS*30*, but also by other transposable elements.

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