IS*26*-Mediated Precise Excision of the IS*26*-*aphA1a* Translocatable Unit

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ABSTRACT **We recently showed that, in the absence of RecA-dependent homologous recombination, the Tnp26 transposase catalyzes cointegrate formation via a conservative reaction between two preexisting IS*26*, and this is strongly preferred over replicative transposition to a new site. Here, the reverse reaction was investigated by assaying for precise excision of the central region together with a single IS*26* from a compound transposon bounded by IS*26*. In a *recA* mutant strain, Tn*4352*, a kanamycin resistance transposon carrying the *aphA1a* gene, was stable. However, loss of kanamycin resistance due to precise excision of the translocatable unit (TU) from the closely related Tn*4352*B, leaving behind the second IS*26*, occurred at high frequency. Excision occurred when Tn*4352*B was in either a high- or low-copy-number plasmid. The excised circular segment, known as a TU, was detected by PCR. Excision required the IS*26* transposase Tnp26. However, the Tnp26 of only one IS*26* in Tn*4352*B was required, specifically the IS*26* downstream of the *aphA1a* gene, and the excised TU included the active IS*26*. The frequency of Tn*4352*B TU loss was influenced by the context of the transposon, but the critical determinant of high-frequency excision was the presence of three G residues in Tn*4352*B replacing a single G in Tn*4352*. These G residues are located immediately adjacent to the two G residues at the left end of the IS*26* that is upstream of the *aphA1a* gene. Transcription of *tnp26* was not affected by the additional G residues, which appear to enhance Tnp26 cleavage at this end.**

IMPORTANCE **Resistance to antibiotics limits treatment options. In Gram-negative bacteria, IS*26* plays a major role in the acquisition and dissemination of antibiotic resistance. IS*257* (IS*431*) and IS*1216*, which belong to the same insertion sequence (IS) family, mobilize resistance genes in staphylococci and enterococci, respectively. Many different resistance genes are found in compound transposons bounded by IS*26*, and multiply and extensively antibiotic-resistant Gram-negative bacteria often include regions containing several antibiotic resistance genes and multiple copies of IS*26*. We recently showed that in addition to replicative transposition, IS*26* can use a conservative movement mechanism in which an incoming IS*26* targets a preexisting one, and this reaction can create these regions. This mechanism differs from that of all the ISs examined in detail thus far. Here, we have continued to extend understanding of the reactions carried out by IS*26* by examining whether the reverse precise excision reaction is also catalyzed by the IS*26* transposase.**

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IS*26* plays a critical role in the dissemination of antibiotic resistance genes in Gram-negative bacteria and is associated with genes conferring resistance to many different classes of antibiotics (1–7). IS*26* belongs to the IS*6* family and is most likely identical to IS*6*. Two other insertion sequences (IS) belonging to the IS*6* family, IS*257* and IS*1216*, are also influential in bringing antibiotic resistance genes into the chromosomes and plasmids of staphylococci (8) and enterococci (9), respectively. Hence, understanding the mechanism of mobilization of resistance genes by IS in this family should enhance our understanding of resistance gene acquisition and stability in both Gram-negative and Gram-positive bacteria.

Movement of IS*26* via RecA-independent transposition was first described more than 3 decades ago (10, 11). Three insertion sequences, IS*26*, IS*15*Δ, and IS*140*, which are now known to be identical to one another, have been shown to form cointegrates between the replicon carrying them and a target replicon (10–13), indicating a replicative mode of transposition. However, homologous recombination was required to resolve the cointegrates, reforming two replicons, each of which now carries a copy of the IS (10, 13). Intramolecular replicative transposition can form a circular molecule containing a single copy of IS*26* together with an adjacent DNA segment (Fig. 1A, top), and for clarity, we recently named this entity a translocatable unit or TU (14). If a TU is integrated at a new site via replicative transposition, a compound transposon will be generated (Fig. 1A). In recombination-proficient (wild-type) cells, the TU could also form a compound transposon if it is incorporated via homologous recombination with a preexisting IS*26* (Fig. 1A). Once an IS*26*-bounded transposon has been generated, the TU can also be formed by homologous recombination between the two directly oriented IS*26*.

However, tandem arrays of a resistance gene interspersed with

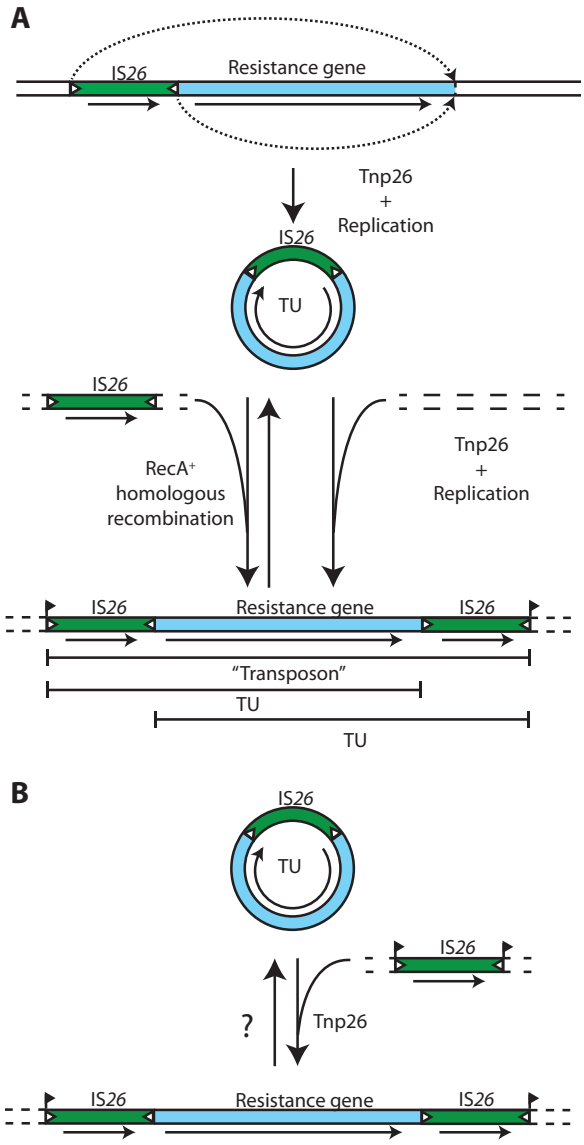


FIG 1 Pathways to TU and transposon formation via Tnp26 replicative transposition or RecA$^+$ homologous recombination (A) or via a conservative Tnp26-dependent, RecA-independent mechanism (B). IS*26* (green box) and the 14-bp inverted repeats of IS*26* (open triangles) are indicated. The position and orientation of target site duplications formed during the insertion of IS*26* are indicated by solid black flags. The extent of the compound transposon and two alternate translocatable units are shown below the schematic representation.

an IS*26* between them (15), or structures with an IS*26* shared between two compound transposons (see Fig. 1 in reference 14), cannot be formed by replicative transposition. To explain these, we recently proposed that when an IS*26* is present in two different molecules (either TU, plasmids or the chromosome) in the same cell, the cointegrate is formed preferentially via a Tnp26-catalyzed reaction between the two IS*26* (Fig. 1B). We found that in *recA* mutant cells, i.e., in the absence of an active homologous recombination system, this reaction occurs in preference to replicative cointegrate formation involving a new site and at higher frequency. It occurs via a conservative mechanism, i.e., an IS*26* is not duplicated, and a target site duplication is not generated (14).

One IS*26*-bounded structure, known as Tn*4352* (2), is often seen in complex configurations with other IS*26*-associated resistance genes (7, 16). Tn*4352* is a 2,681-bp compound transposon consisting of directly oriented copies of IS*26* flanking the kanamycin and neomycin resistance determinant *aphA1a* (Fig. 2A) (2). The *aphA1a* gene encodes the aminoglycoside-3'-phosphotransferase APH(3')-Ia. A variant form that includes two additional G residues adjacent to the IS*26* located upstream of *aphA1a* (Fig. 2C) was previously identified in the A/C$_2$ plasmid pRMH760 (GenBank accession number KF976462) and named Tn*4352*B (17). Tn*4352*B has since been found in an additional three A/C$_2$ plasmids in GenBank (accession nos. FJ621589, CP009409, and CP009414).

Here, whether Tnp26-mediated excision of a TU from Tn*4352* and Tn*4352*B, as shown in Fig. 1B, can occur was examined, and the features required for TU loss have been determined.

RESULTS

Tn*4352* is stable, while Tn*4352*B is not. IS*26*-bounded structures, such as Tn*4352* (IS*26*-*aphA1a*-IS*26*), conferring resistance to kanamycin and neomycin, appear to be stable. When *recA* mutant *Escherichia coli* cells containing plasmid pRMH991 (Ap^r Km^r), a pUC19 derivative containing Tn*4352* in a DNA fragment from a large *E. coli* plasmid, pDGO100 (18), were grown without kanamycin selection, no kanamycin-susceptible (Km^s) cells were detected among 1,500 colonies screened over five cycles of serial subculture (100 colonies screened at each cycle in each of three independent experiments) (Fig. 2A). However, we fortuitously observed that kanamycin resistance was rapidly lost when the plasmid was pRMH761 (Ap^r Km^r), a pUC19 derivative containing Tn*4352*B in a DNA fragment from pRMH760 (19). All cells in the culture retained resistance to ampicillin, confirming that the plasmid had not been lost. After approximately 22 generations (one cycle of overnight growth), less than 50% of cells in the culture were resistant to kanamycin. After five cycles of repeated subculture, less than 1% of cells were resistant. This result was reproducible, and the averages of five independent experiments are shown in Fig. 2B. When the same cells were grown under kanamycin selective pressure, all cells retained resistance to kanamycin (averages of three independent experiments shown in Fig. 3A).

Tn*4352*B differs from the more common Tn*4352* form by the presence of two additional G nucleotides directly adjacent to the left inverted repeat (IRl) of the right-hand IS*26* (Fig. 2C). However, the genetic context surrounding Tn*4352* in pRMH991, which is in *merA* of pDU, is also different from that surrounding Tn*4352*B in pRMH761, which is in *tniA* of In34. Whether the difference in the ability to lose *aphA1a*, and hence kanamycin resistance, was due to the context of the transposon or to the two additional G nucleotides was examined by exchanging the central segment, forming Tn*4352* in *tniA* (pRMH976) and Tn*4352*B in *merA* (pRMH992). Excision of the TU was detected from Tn*4352*B in *merA* (pRMH992 [Fig. 2A]), but when Tn*4352* was in *tniA* (pRMH976), no Km^s colonies were detected even after 5 days of serial subculture (Fig. 2B). Hence, the two G's were essential for TU loss to be detected. The difference in the kinetics of excision of the TU from Tn*4352*B in *merA* compared to Tn*4352*B in *tniA* indicates that other factors in the sequence flanking Tn*4352*B may also be important.

The Tn*4352*B TU is excised precisely. Plasmid DNA extracted after a single cycle (22 generations) of growth of cells containing

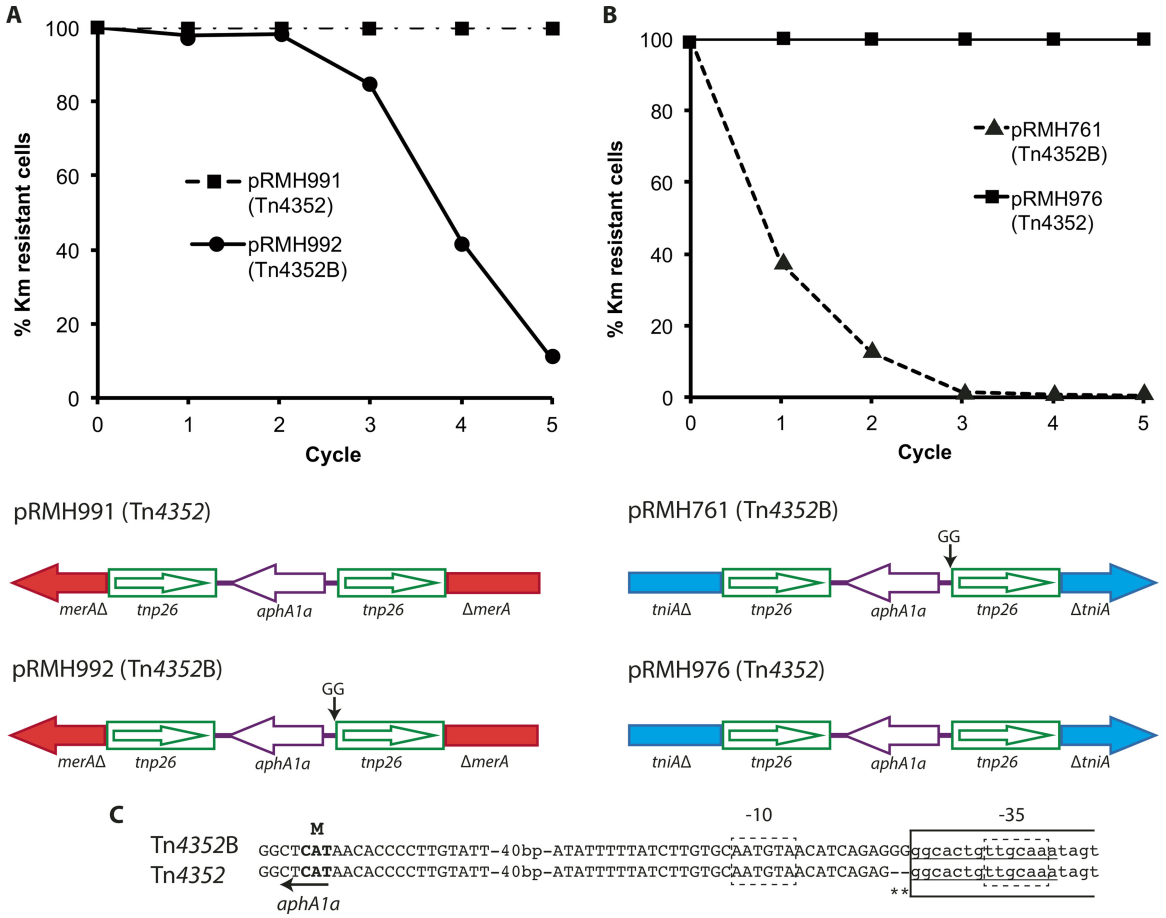


FIG 2 TU excision from Tn*4352* and Tn*4352B* in two contexts. (A) TU loss from Tn*4352* and Tn*4352B* in *merA*. (B) TU loss from Tn*4352*B and Tn*4352* in *tniA*. IS*26* is represented by a green box with an arrow indicating the position and orientation of *tnp26*. The purple arrow indicates the position and orientation of *aphA1a*. GG marks the position of the additional GC base pairs in Tn*4352*B. (C) Partial sequence alignment of Tn*4352* and Tn*4352*B. The boxed bases belong to IS*26*, and underlined bases mark the 14-bp left inverted repeat of IS*26*. Dashed boxes mark the *aphA1a* −10 and −35 promoters. Bold bases indicate the *aphA1a* start codon.

pRMH761 (Tn*4352*B) without kanamycin selection was digested with BamHI. In addition to the ~8.8-kb BamHI fragment containing Tn*4352*B (Fig. 3B) and the 2.7-kb pUC19 backbone expected from pRMH761, a smaller BamHI fragment of approximately 7.0 kb was also present (Fig. 3C). Plasmid DNA from 10 individual Ap^r Km^s colonies contained only the smaller ~7.0-kb BamHI fragment. Sequencing revealed that only a single copy of IS*26* was retained in these plasmids. Hence, a single IS*26* and the complete central region containing *aphA1a*, i.e., the predicted TU, had been removed precisely (Fig. 3B).

To detect the excised circular TU, primers facing outwards from *aphA1a* (aphA1-A and aphA1-B) were used. These primers would generate a 1,197-bp PCR amplicon if the TU were present in a circular form (Fig. 3F). Amplicons of the predicted size were generated from DNA extracted from cultures of cells containing pRMH761 (Tn*4352*B) grown either with or without kanamycin selection (Fig. 3F). The amplicon from five independent experiments was sequenced, and in all cases, it contained a single IS*26* flanked by the ends of the central segment, i.e., the boundaries were as expected if the circular TU was excised precisely.

Furthermore, while the excised TU could be detected via PCR in plasmid DNA from cultures of cells containing Tn*4352*B in both the *tniA* and *merA* contexts, no PCR product was detected from cells containing Tn*4352* in either context.

TU excision requires Tnp26. The instability of kanamycin resistance in plasmids containing Tn*4352*B was observed in a *recA* mutant background, suggesting that excision was catalyzed by the IS*26*-encoded transposase Tnp26. To establish the role of Tnp26, frameshift mutations that truncate the Tnp26 protein to 30 amino acids were introduced into both copies of IS*26* in pRMH761 to form pRMH990 (Fig. 3E). In three independent experiments, no kanamycin-susceptible cells were detected after five serial growth cycles (approximately 110 generations) of cells containing pRMH990 in the absence of kanamycin (Fig. 3D). Furthermore, the excised TU could not be detected by PCR. Hence, Tnp26 is required.

Additional G nucleotides do not increase *tnp26* transcription. The possibility that the additional G nucleotides upstream of *tnp26* in Tn*4352*B caused increased transcription of *tnp26* was considered. The level of *tnp26* expression was quantified via reverse transcription-quantitative PCR (RT-qPCR) analysis of clones containing an IS*26* with (pRMH974) or without (pRMH973) the two G nucleotides adjacent to it. No significant difference between *tnp26* transcript levels was observed in three

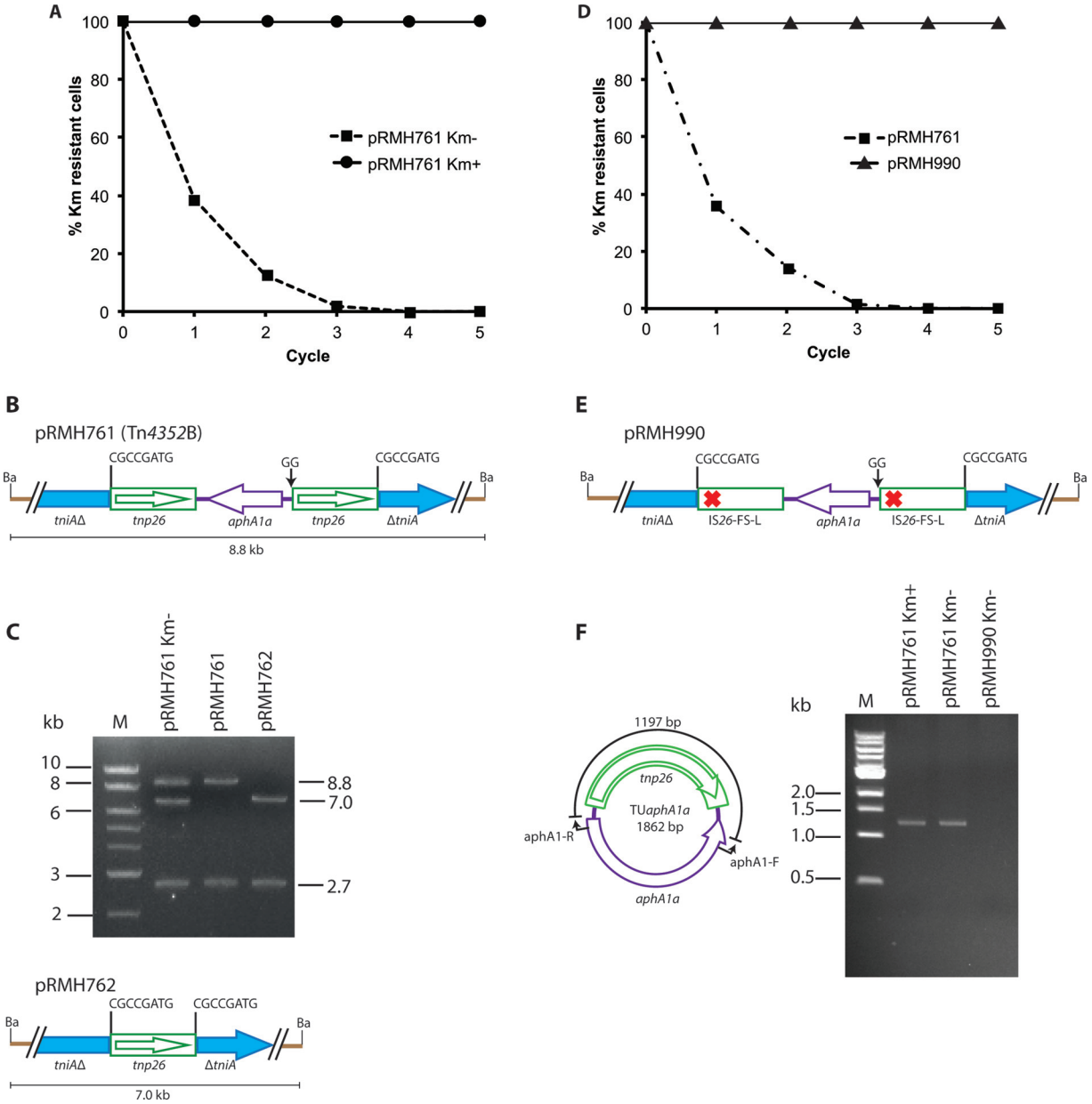
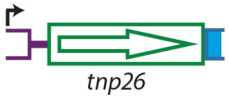
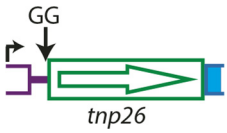
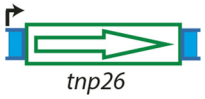
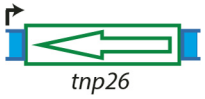


FIG 3 TU loss from Tn*4352*B. (A) Proportion of kanamycin-resistant cells from cultures containing pRMH761 grown with kanamycin selection (KM+) or without kanamycin selection (Km−). (B) Configuration of pRMH761 and pRMH762. IS*26* is represented by a green box with an arrow indicating the position and orientation of *tnp26*. The purple arrow indicates the position and orientation of *aphA1a*. Ba denotes a BamHI restriction site, and the size of the BamHI restriction fragment is marked below the schematic representation. The 8-bp duplication of the *tniA* target is shown above the schematic representation. (C) BamHI digestion of plasmid DNA from cells containing pRMH761 grown without kanamycin selection (pRMH761 Km−) and with kanamycin selection (pRMH761) and of a representative kanamycin-sensitive derivative (pRMH762). The sizes of the restriction fragments (in kilobases) are marked to the right of the gel, and the sizes of relevant bands in the marker (M) are shown to the left of the gel. (D) Proportion of kanamycin-resistant cells from cultures containing pRMH761 or a pRMH761 derivative with frameshifts in both copies of *tnp26* (pRMH990) grown without kanamycin selection. (E) Schematic of pRMH990. Green boxes represent IS*26*, and a thick red cross indicates the position of the frameshift (left frameshift [FS-L]) in *tnp26*. (F) PCR detection of a circular TU. The positions of the primers and the expected amplicon size are marked on the circular schematic. The sizes of relevant bands in the marker are shown to the left of the gel.

independent experiments (Table 1). This correlates with no significant difference in the frequency of cointegrate formation between these constructs and R388::IS*26* (Table 1). Hence, the additional G nucleotides do not influence either *tnp26* expression or the integration frequency. The level of *tnp26* transcripts and transposition frequency were also tested for a fragment containing the left-hand IS*26* from Tn*4352*B (pRMH975) to determine whether both *tnp26* from Tn*4352*B were expressed equally. There was no significant difference to the right-hand IS*26* clones with or without the additional G nucleotides (Table 1).

The level of *tnp26* transcription and the transposition frequency of additional constructs containing a *tniA*-flanked

TABLE 1 Features of single IS*26* constructs in different contexts

Plasmid	Structure[a]	Mean transposition frequency (range)[b]	*tnp26* expression[c]
pRMH973		3.1×10^{-4} (1.9×10^{-4}– 5.1×10^{-4})	1
pRMH974		3.2×10^{-4} (1.9×10^{-4}–4.1×10^{-4})	0.91 (0.57–1.29)
pRMH975		4.9×10^{-4} (3.5×10^{-4}–7.1×10^{-4})	0.87 (0.77–1.05)
pRMH977		2.1×10^{-4} (1.9×10^{-4}–2.4×10^{-4})[d]	1.09 (0.64–1.40)
pRMH979		3.4×10^{-4} (2.9×10^{-4}–3.8×10^{-4})	0.63 (0.50–0.73)

[a] The location of P_{lac} is shown by the small black bent arrow.
[b] The transposition frequency into R388::IS*26* is expressed as the number of cointegrates per transconjugant. The transposition frequency was determined in three independent experiments.
[c] *tnp26* expression relative to *tnp26* expression in pRMH973. *tnp26* expression was determined in three independent experiments.
[d] Previously reported in Harmer et al. (14).

IS*26* with *tnp26* in both the same (pRMH977) and opposite (pRMH979) orientation with respect to the pUC promoter were also determined. While the level of *tnp26* transcription was consistently higher (Table 1) in three independent experiments when P_{lac} could potentially drive transcription of *tnp26* (pRMH977), the difference between the two orientations was not statistically significant. There was also no significant difference in transposition frequency between the two orientations (Table 1). Hence, the level of *tnp26* expression does not appear to be responsible for the difference in excision frequency.

TU excision requires only one active Tnp26. To determine whether an active transposase produced by both or only one of the IS*26* in Tn*4352*B is required to excise the TU, individual frameshift mutations were introduced into only one of the *tnp26* of pRMH761. When the right-hand IS*26* was disrupted (pRMH995 [Ap^r Km^r]), kanamycin resistance was lost at a rate similar to that seen for the parent pRMH761 (Table 2). In contrast, kanamycin-susceptible cells were not detected even after 5 days of subculture when the left-hand IS*26* *tnp26* was inactivated in pRMH994 (Table 2). The circular TU excised from pRMH995 was detected via PCR, but no amplicon was seen for pRMH994.

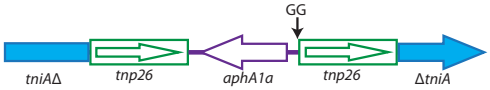
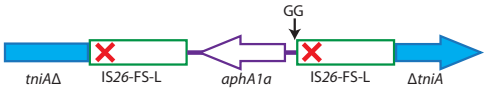
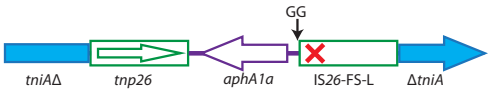
To determine which copy of IS*26* was incorporated into the TU, the sequence of the IS*26* remaining in the derivatives of pRMH995 recovered from five independent kanamycin-sensitive colonies was determined. In all cases, the additional 4 bp in the *tnp26* mutant was present. Hence, the TU had been formed from the active left-hand IS*26* and the *aphA1a*-containing central fragment and the sequence of the amplicon derived from the TU confirmed this.

TU loss from low-copy-number plasmids. To eliminate the possibility that TU excision was due to the high copy number or other properties of the pUC19-based constructs tested so far, the low-copy-number IncW plasmids R388::Tn*4352*B and R388::Tn*4352* were tested. Excision of the TU from R388::Tn*4352*B was detected after growth for one cycle without antibiotic selection (85% of cells were resistant). After 5 days, only 10% of cells were kanamycin resistant (Table 3). Loss of kanamycin resistance from cells containing R388::Tn*4352* was not detected, even after five cycles of growth without selection (Table 3).

The large A/C_2 plasmid pRMH760, from which the Tn*4352*B-containing fragment in pRMH761 was derived, was transferred into *recA* mutant *E. coli* DH5α cells and grown without antibiotic selection. In this instance, loss of *aphA1a* was assessed using neomycin resistance instead of kanamycin resistance, as pRMH760 also contains the *aadB* resistance cassette conferring resistance to kanamycin, gentamicin, and tobramycin (20). Neomycin-susceptible cells were not detected after one cycle (22 generations) of growth but were found after two cycles, and after five cycles of serial subculture, only 6% of cells retained Tn*4352*B (Table 3). Loss of neomycin resistance was also assessed using cells carrying pDGO100, the A/C_2 plasmid carrying Tn*4352* in *merA* of pDU. No neomycin-susceptible colonies were detected after five cycles of growth of pDGO100-containing cells without selection (Table 3), consistent with the results obtained when Tn*4352* was cloned into pUC19.

Excision of a plasmid from R388-pUC19 cointegrates. The potential product of excision from cointegrates formed between R388::IS*26* (Tp^r) and pRMH973 (Ap^r) or pRMH974 (Ap^r) carry-

TABLE 2 Testing TU excision from Tn*4352*B derivatives with inactive Tnp26

Plasmid	Structure	% of Km^r cells[a]				
		Day 1	Day 2	Day 3	Day 4	Day 5
pRMH761		39.0	12.3	1.7	<1	<1
pRMH990		100.0	100.0	100.0	100.0	100.0
pRMH994		100.0	100.0	100.0	100.0	100.0
pRMH995		43.7	21.0	6.7	2.3	<1

[a] Determined by picking and patching 100 colonies. Numbers are the averages of three independent experiments.

ing a single IS*26* (Table 1) would contain pUC19 and hence would replicate. As replication amplifies the excised product, this provides a means to detect low-frequency excision events. Plasmid DNA extracted from cells grown overnight without selection was transformed into *recA* mutant *E. coli* cells. Ap^r colonies were screened for resistance to trimethoprim, and Ap^r Tp^s cells should contain only the pUC19-based plasmid, indicating excision. These plasmids were detected at relatively high frequency from R388-pRMH974 cointegrates, which contain the additional G nucleotides derived from Tn*4352*B. After five cycles of growth without selection, between 79 and 85% of transformed cells contained only the excised plasmid, in three independent experiments (Table 4). Restriction mapping, PCR, and sequencing of five plasmids recovered in each of the three independent experiments revealed that their structures were identical to that of the parental pUC19-based plasmid, i.e., the plasmid had been excised precisely.

Plasmid DNA recovered from cells containing the R388-pRMH973 cointegrate (which does not contain the additional G nucleotides) after five cycles of growth in each of the three independent experiments (Table 4) was also examined. Two excised Ap^r Tp^s plasmids were recovered by transformation from two of the three plasmid DNA samples (total of four plasmids recovered) after screening 1,000 Ap^r transformants from each sample. The sequences of these plasmids were identical to the sequence of the parental pUC19-based plasmid. This demonstrates that a TU can be excised from IS*26*-bounded structures without the additional G nucleotides, but at very low frequency.

DISCUSSION

IS*26* is able to mobilize adjacent DNA segments via intramolecular replicative transposition and create a new composite transposon by intermolecular replicative transposition (Fig. 1A). A distinct RecA-independent, Tnp26-dependent mode of IS*26* movement is conservative (14). It involves the pairing of preexisting copies of IS*26* and can account for the formation of regions containing multiple copies of IS*26* interspersed with the same or different DNA segments. In these structures, compound transposons share an IS*26*. In the current study, we have confirmed experimentally that the reverse of this conservative reaction rarely occurs by showing that the IS*26*-bounded transposon Tn*4352* is very stable. This would ensure the stable maintenance of IS*26*-bounded transposons. This observation is consistent with the previously reported requirement for homologous recombination to resolve cointegrates formed by IS*26* (10–13), and although cointegrate resolution was detected here in a *recA* mutant strain, it was extremely rare. However, a TU made up of a single IS*26* and the

TABLE 3 TU loss from low-copy-number plasmids in *recA* mutant *E. coli*

Plasmid	Transposon	Expt[a]	% of Km^r cells[b] after the following cycle (no. of generations):				
			1 (22)	2 (44)	3 (66)	4 (88)	5 (110)
R388::Tn*4352*B	Tn*4352*B	1	83	46	23	15	7
		2	88	54	30	15	12
		3	92	58	36	18	8
R388::Tn*4352*	Tn*4352*	1–3	100	100	100	100	100
pRMH760	Tn*4352*B	1	100	84	53	21	5
		2	100	87	52	24	7
		3	100	86	49	24	6
pDGO100	Tn*4352*	1–3	100	100	100	100	100

[a] Three independent experiments were performed.
[b] Measured as the number of kanamycin-resistant cells (or neomycin-resistant cells in the case of pRMH760 and pDGO100) divided by the number of total cells. The proportions reported were determined by picking and patching 100 colonies.

TABLE 4 TU excision from pRMH973 and pRMH974-derived cointegrates

R388::IS*26* cointegrate	% of Ap^r Tp^s colonies[a]				
	Cycle 1	Cycle 2	Cycle 3	Cycle 4	Cycle 5
pRMH974-1	2	5	20	50	85
pRMH974-2	3	7	26	57	79
pRMH974-2	3	4	21	46	82
pRMH973-1	0	0	0	0	0
pRMH973-2	0	0	0	0	1
pRMH973-3	0	0	0	0	1

[a] Determined by screening 100 Ap^r colonies from each cycle.

aphA1a segment can be formed from Tn*4352*B at a surprisingly high frequency in a conservative, Tnp26-dependent, *recA*-independent manner, and this will allow the reactions catalyzed by Tnp26 to be explored in more detail.

The presence of a run of five G's was critical for high-frequency excision to occur, and it will be interesting to determine the effects of four G residues in this position. Whereas both IS*26* must encode an active transposase for integration to occur at high frequency (14), only an active left-hand IS*26* (as shown in Table 2) is required for TU formation, and this IS is incorporated into the excision product. Hence, only the left end of the right-hand IS*26* is likely to be involved in the excision event. The position of the extra G nucleotides, directly adjacent to this end in Tn*4352*B, suggests that they influence the recognition of the outer end of the IS by Tnp26. The five G's could potentially lead to stiffening or straightening of the DNA secondary structure relative to that when only three G's are present, bringing the active site of Tnp26 into the correct position relative to the outer end for the cleavage reaction to occur. This possibility requires further exploration.

Though the precise mechanics of the Tnp26-catalyzed reactions reported here remain to be determined, it is clear that in addition to the well-understood replicative transposition, IS*26*-bounded structures can be mobilized by a mechanism that differs from those of all of the ISs examined in detail thus far. This mode of movement is likely to also apply to other members of the IS*26* family, including IS*257* and IS*1216* that play a major role in the mobilization of antibiotic resistance genes in Gram-positive bacteria.

MATERIALS AND METHODS

Bacterial strains and media. *E. coli* DH5α (*supE44* Δ*lacU169* [ϕ80 *lacZ*ΔM15] *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*) was used to propagate plasmids. *E. coli* UB1637 (*lys his trp lac recA* Sm^r) was used as a recipient in transposition experiments. Bacteria were routinely cultured at 37°C in Luria-Bertani (LB) medium or on LB agar. Mueller-Hinton agar was used to select for resistance to trimethoprim. The following antibiotics (Sigma) were added at the indicated concentrations: ampicillin, 100 μg ml^{-1}; kanamycin, 50 μg ml^{-1}; neomycin, 50 μg ml^{-1}; streptomycin, 25 μg ml^{-1}; and trimethoprim, 10 μg ml^{-1}.

DNA manipulation. Plasmid DNA for restriction analysis and cloning was isolated by an alkaline lysis method (21). Digestion with restriction enzymes and cloning was performed using standard methods as previously described (14). PCR was performed using standard conditions (14). The sequences for all primers used in this study are listed in Table 5. PCR products were purified for sequencing via gel extraction using an EconoSpin DNA column (Epoch Life Sciences). Routine sequencing was performed at the Australian Genome Research Facility (Sydney, Australia).

Quantitative real-time PCR was performed as described previously (22), using primers RH1464 and RH1465 (Table 5) to detect expression of *tnp26*. bla_{TEM-1}, constitutively expressed from the plasmid backbone, was used as an endogenous control (primers RH1466 and RH1467). Real-time PCR was performed in triplicate on independent biological replicate samples.

TABLE 5 Primers used in this study

Primer	Sequence (5′–3′)[a]
aphA1-A	CAAAAATATGGTATTGATAATCCTG
aphA1-B	TATACCCATATAAATCAGCATCC
RH1451	TTCGGTGGATCCACCCAGATACGGCTGATGTC
RH1452	CACACGGGATCCTTCGTTCCTGGTCGATTTTC
RH1453	CTTCCCGGATCCTCGATAGATTGTCGCACCTG
RH1454	CATCTTGGATCCCTGCCTCGGTGAGTTTTCTC
RH1462	GCGGCGGGATCCAAGGAAGCGCTGGAAAAAGT
RH1463	GCCCCTGGATCCTCGTCAGGTAGGGGAACAAC
RH1464	ACCTTTGATGGTGGCGTAAG
RH1465	TACCGGAACAACGTGATTGA
RH1466	AAGCCATACCAAACGACGAG
RH1467	TTGCCGGGAAGCTAGAGTAA
RH1471	CCGCTCCAAAAACTATCCAC
RH1472	ATCGGAAATGGTTGTGAAGC

[a] BamHI restriction sites incorporated into the 5′ end of the primer are underlined.

Plasmids. The plasmids used in this study are listed in Table 6. pRMH760 (19) and pDGO100 (18) are naturally occurring A/C$_2$ plasmids that carry Tn*4352*B and Tn*4352*, respectively. R388 is an IncW plasmid that conjugates at high frequency, and R388::IS*26* was described previously (14). pRMH761 (Ap^r Km^r Nm^r) has been described previously (14, 17) and consists of an 8.8-kb BamHI fragment from pRMH760 (bases 117841 to 126672 in GenBank accession number KF976462) cloned into pUC19 (Ap^r). The BamHI fragment contains Tn*4352*B (Km^r Nm^r) in the *tni* gene of a class 1 integron. pRMH762 is a kanamycin-sensitive derivative of pRMH761 containing only a single IS*26* and was described previously (14). Plasmid pRMH991 was generated from pDGO100 (18), which contains Tn*4352* in *merA* of pDU. Tn*4352* was amplified together with 566 bp of *merA* on the left and 1,000 bp on the right using primers RH1462 and RH1463 (4,253-bp product) and cloned into pUC19 to generate pRMH991. The sequence of the cloned fragment has been deposited in GenBank under accession number KT207463.

A plasmid with Tn*4352* in the pRMH761 genetic context (*tniA* of In34) was constructed by ligating the 1.8-kb SwaI fragment from pRMH991 with the 9.6-kb SwaI fragment from pRMH761 to generate plasmid pRMH976. The reciprocal construct (pRMH992), with Tn*4352*B in the pDGO100 *merA* context, was generated by ligating the 1.8-kb SwaI fragment from pRMH761 to the 5.1-kb SwaI fragment from pRMH991.

Plasmid construction. A derivative of pRMH761, pRMH990, with a frameshift disrupting the transposase reading frames in both *tnp26* of Tn*4352*B was generated as follows. Five micrograms of purified pRMH761 plasmid DNA was digested with 10 U of BsiWI for 2 h at 55°C, and the ends of the resulting 1.8-kb and 9.6-kb fragments were filled with T4 DNA polymerase (New England Biolabs) according to the manufacturer's instructions and religated. Plasmid DNA from colonies selected with kanamycin and ampicillin was mapped by restriction digestion with PstI and NdeI to confirm that the end-filled fragments were in the correct orientation and with BsiWI and SnaBI to confirm that the end of the BsiWI site had been filled. This introduced a 4-bp duplication of bases 114 to 117 relative to the left end of each IS*26* and bases 52 to 55 relative to the first base of the *tnp26* gene. For pRMH990, this was confirmed by sequencing the left and right IS*26* with primers RH1451 and RH1453 on the left and primers RH1452 and RH1454 on the right. The 4-bp duplication introduced a frameshift in the *tnp26* gene leading to premature termination at a stop codon (bases 154 to 156 in IS*26*), forming only a 30-amino-acid (aa) peptide.

To generate transposons with a frameshift in only the left or right *tnp26* of Tn*4352*B, pRMH761 and pRMH990 were digested with 10 U of

TABLE 6 Plasmids used in this study

Plasmid	Description	Transposon	Resistance phenotype[a]	Reference
pRMH760	A/C_2 plasmid containing Tn*4352*B in *tniA*	Tn*4352*B	Ap Cm Gm Km Nm Su Tb Tp	19
pRMH761	8.8-kb BamHI fragment of pRMH760 containing Tn*4352*B cloned into pUC19	Tn*4352*B	Ap Km Nm	14
pRMH976	pRMH761 derivative containing Tn*4352* in *tniA*[b]	Tn*4352*	Ap Km Nm	This study
pRMH990	pRMH761 derivative with frameshifts in both *tnp26*[c]		Ap Km Nm	This study
pRMH994	pRMH761 derivative with a frameshift in the right *tnp26*		Ap Km Nm	This study
pRMH995	pRMH761 derivative with a frameshift in the left *tnp26*		Ap Km Nm	This study
pDGO100	A/C_2 plasmid containing Tn*4352* in pDU *merA*	Tn*4352*	Ap Cm Gm Km Nm Su Tb Tp	18
pRMH991	4.3-kb fragment of pDGO100 containing Tn*4352* in pDU *merA* cloned into pUC19[d,e]	Tn*4352*	Ap Km Nm	This study
pRMH992	pRMH991 derivative containing Tn*4352*B in pDU *merA*[f]	Tn*4352*B	Ap Km Nm	This study
R388::IS*26*	R388 with IS*26*[g]		Su Tp	14
R388::Tn*4352*B	R388 containing Tn*4352*B[h]	Tn*4352*B	Km Nm Su Tp	14
R388::Tn*4352*	R388 containing Tn*4352*[h]	Tn*4352*	Km Nm Su Tp	This study
pRMH973	Right IS*26* from pRMH976 cloned into pUC19[i]		Ap	This study
pRMH974	Right IS*26* from pRMH761 cloned into pUC19[i]		Ap	This study
pRMH975	Left IS*26* from pRMH761 cloned into pUC19[i]		Ap	This study
pRMH977	pRMH762 derivative[i,j]		Ap	14
pRMH979	pRMH762 derivative[j,k]		Ap	14

[a] Ap, ampicillin; Cm, chloramphenicol; Gm, gentamicin; Km, kanamycin; Nm, neomycin; Su, sulfamethoxazole; Tb, tobramycin; Tp, trimethoprim.
[b] 1.8-kb central SwaI Tn*4352*B fragment replaced with Tn*4352* from pDGO100.
[c] Frameshift generated by end filling the BsiWI site and duplicating 116 to 119 bp from the left end of IS*26* as shown in Fig. 5 of Harmer et al. (14).
[d] Tn*4352* from pDGO100 cloned into pUC19 in the same orientation as pRMH761, with the 5′ end of *tnp26* closest to P_{lac}.
[e] Bases 75 to 4320 from GenBank accession number KT207463.
[f] 1.8-kb SwaI Tn*4352* fragment replaced with Tn*4352*B from pRMH761.
[g] IS*26* 8-bp duplication of bases 26745 to 26752 in R388 (GenBank accession number BR000038).
[h] Tn*4352*B together with 8-bp duplication of bases 26745 to 26752 in R388 (GenBank accession number BR000038).
[i] Cloned insert in the opposite orientation to pRMH761, with the 5′-end *tnp26* closest to P_{lac} in pUC19.
[j] IS*26* together with bases 119362 to 119454 and 122137 to 122225 from GenBank accession number KF976462.
[k] Cloned insert in the same orientation as in pRMH761, with the 3′ end of *tnp26* closest to P_{lac} in pUC19.

SwaI. The 1.8-kb fragment internal to Tn*4352*B and the 9.6-kb fragment from each plasmid were separated and purified by electrophoresis through a 0.7% agarose gel. Ligating the 1.8-kb fragment of each plasmid with the 9.6-kb fragment of the other generated pRMH994 with a frameshift in the left-hand IS*26* *tnp26* and pRMH995 with a frameshift in the right-hand IS*26* *tnp26*. The presence of the mutation was confirmed by sequencing both ISs in each construct.

Plasmids containing a single IS*26* were constructed via PCR amplification followed by cloning into pUC19 such that the 5′ end of *tnp26* was closest to P_{lac}. The right-hand IS*26* from pRMH761 (Tn*4352*B) and pRMH976 (Tn*4352*) was amplified using primers RH1453 and RH1451 and generated constructs pRMH974 and pRMH973, respectively. The left-hand IS*26* from pRMH761 amplified using primers RH1454 and RH1452 and cloned into pUC19 as described above generated pRMH975. A construct containing a *tniA*-flanked IS*26* was amplified together with 89 bp of flanking sequence on the left and 116 bp on the right from pRMH762 using primers RH1451 and RH1452 and cloned into pUC19 with either the 5′ or 3′ end of *tnp26* closest to P_{lac} to generate plasmids pRMH977 and pRMH979, respectively.

Quantification of TU loss. A minimum of three independent experiments were performed for each plasmid tested. Starter cultures of *recA* mutant *E. coli* DH5α cells containing the appropriate plasmid were grown overnight at 37°C in LB with kanamycin selection. One hundred microliters of a 10^{-5} dilution of starter culture (approximately 1,000 cells) was used to inoculate cultures with or without kanamycin (50 μg ml^{-1}) or neomycin (50 μg ml^{-1}) to select for retention of *aphA1a* and grown overnight (16 h [approximately 22 generations]). Cultures were diluted, and a fresh culture was inoculated with ~1,000 cells; this was performed daily for five cycles. To detect kanamycin-resistant and kanamycin-sensitive cells, the culture was serially diluted and plated in duplicate onto LB agar supplemented with both ampicillin and kanamycin (to select for pUC19 with the *aphA1a*-containing Tn*4352*B/Tn*4352*) and ampicillin alone to determine the total plasmid-containing cells. The percentage of kanamycin-resistant cells was determined by dividing the total number of kanamycin-resistant cells by the total number of cells on the ampicillin plate. To confirm the colony counts, 100 colonies from each overnight culture were patched onto LB agar supplemented with ampicillin or ampicillin and kanamycin. In all instances, the patch counts were consistent with the colony counts. Plasmid DNA was prepared from each culture and BamHI digested to determine the fraction containing the *aphA1a*-containing fragment.

A different strategy was used to detect excision of the TU from cointegrates formed between R388::IS*26* (Su^r Tp^r) and either pRMH974 (Ap^r) or pRMH973 (Ap^r). As the excised TU in this instance contains the pUC19 backbone and is hence able to replicate, excision could not be detected using the strategy employed for TU*aphA1a*. Instead, plasmid DNA was extracted from overnight cultures and 100 ng of plasmid DNA was transformed by electroporation into *E. coli* DH5α cells. One hundred Ap^r colonies from each transformation were patched onto plates containing either ampicillin (100 μg ml^{-1}) or trimethoprim (10 μg ml^{-1}). Ap^r Tp^s colonies carry the pUC19-based plasmid that had been excised from the R388 cointegrate. The frequency of loss was calculated as the number of Ap^r Tp^s transformants divided by the total number of Ap^r cells.

Transposition assays. Standard conduction assays were performed as described previously (14). Cointegrates were recovered, and their structures were verified as described previously (14).

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