IS26-Mediated Precise Excision of the IS26-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 IS26, 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 IS26 from a compound transposon bounded by IS26. In a recA mutant strain, Tn4352, 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 Tn4352B, leaving behind the second IS26, occurred at high frequency. Excision occurred when Tn4352B 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 IS26 transposase Tnp26. However, the Tnp26 of only one IS26 in Tn4352B was required, specifically the IS26 downstream of the aphA1a gene, and the excised TU included the active IS26. The frequency of Tn4352B 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 Tn4352B replacing a single G in Tn4352. These G residues are located immediately adjacent to the two G residues at the left end of the IS26 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, IS26 plays a major role in the acquisition and dissemination of antibiotic resistance. IS257 (IS431) and IS1216, 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 IS26, and multiply and extensively antibiotic-resistant Gram-negative bacteria often include regions containing several antibiotic resistance genes and multiple copies of IS26. We recently showed that in addition to replicative transposition, IS26 can use a conservative movement mechanism in which an incoming IS26 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 IS26 by examining whether the reverse precise excision reaction is also catalyzed by the IS26 transposase.

S26 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). IS26 belongs to the IS6 family and is most likely identical to IS6. Two other insertion sequences (IS) belonging to the IS6 family, IS257 and IS1216, 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 IS26 via RecA-independent transposition was first described more than 3 decades ago (10, 11). Three insertion sequences, IS26, IS15Δ, and IS140, 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 IS26 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 IS26 (Fig. 1A). Once an IS26-bounded transposon has been generated, the TU can also be formed by homologous recombination between the two directly oriented IS26. However, tandem arrays of a resistance gene interspersed with...
One IS26-bounded structure, known as Tn4352 (2), is often seen in complex configurations with other IS26-associated resistance genes (7, 16). Tn4352 is a 2,681-bp compound transposon consisting of directly oriented copies of IS26 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 IS26 located upstream of aphA1a (Fig. 2C) was previously identified in the A/C2 plasmid pRMH760 (GenBank accession number KP976462) and named Tn4352B (17). Tn4352B has since been found in an additional three A/C2 plasmids in GenBank (accession nos. FJ621589, CP009409, and CP009414).

Here, whether Tnp26-mediated excision of a TU from Tn4352 and Tn3352B, as shown in Fig. 1B, can occur was examined, and the features required for TU loss have been determined.

**RESULTS**

**Tn4352 is stable, while Tn3352B is not.** IS26-bounded structures, such as Tn4352 (IS26-aphA1a-IS26), conferring resistance to kanamycin and neomycin, appear to be stable. When recA mutant *Escherichia coli* cells containing plasmid pRMH991 (Ap’ Km’), a pUC19 derivative containing Tn4352 in a DNA fragment from a large *E. coli* plasmid, pDGO100 (18), were grown without kanamycin selection, no kanamycin-resistant (Km+) cells were detected among 1,500 colonies screened over five cycles of serial subculture (100 colonies screened 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’ Km’), a pUC19 derivative containing Tn4352B 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. Kanamycin-selective 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).

Tn4352B differs from the more common Tn3352 form by the presence of two additional G nucleotides directly adjacent to the left inverted repeat (IRL) of the right-hand IS26 (Fig. 2C). However, the genetic context surrounding Tn3352 in pRMH991, which is in merA of pDU, is also different from that surrounding Tn4352B 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 Tn3352 in tniA (pRMH976) and Tn3352B in merA (pRMH992) (Fig. 2A). Excision of the TU was detected from Tn3352B in merA (pRMH992 [Fig. 2A]), but when Tn3352 was in tniA (pRMH976), no Km+ 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 Tn3352B in merA compared to Tn3352B in tniA indicates that other factors in the sequence flanking Tn3352B may also be important.

**The Tn3352B TU is excised precisely.** Plasmid DNA extracted after a single cycle (22 generations) of growth of cells containing

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**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). IS26 (green box) and the 14-bp inverted repeats of IS26 (open triangles) are indicated. The position and orientation of target site duplications formed during the insertion of IS26 are indicated by solid black flags. The extent of the compound transposon and two alternate translocatable units are shown below the schematic representation.

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an IS26 between them (15), or structures with an IS26 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 IS26 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 IS26 (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 IS26 is not duplicated, and a target site duplication is not generated (14).
pRMH761 (Tn4352B) without kanamycin selection was digested with BamHI. In addition to the ~8.8-kb BamHI fragment containing Tn4352B (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 Apr Kms colonies contained only the smaller ~7.0-kb BamHI fragment. Sequencing revealed that only a single copy of IS26 was retained in these plasmids. Hence, a single IS26 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 (Tn4352B) 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 IS26 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 Tn4352B in both the tniA and merA contexts, no PCR product was detected from cells containing Tn4352 in either context.

**TU excision requires Tnp26.** The instability of kanamycin resistance in plasmids containing Tn4352B was observed in a recA mutant background, suggesting that excision was catalyzed by the IS26-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 IS26 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 Tn4352B 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 IS26 with (pRMH974) or without (pRMH973) the two G nucleotides adjacent to it. No significant difference between tnp26 transcript levels was observed in three
independent experiments (Table 1). This correlates with no significant difference in the frequency of cointegrate formation between these constructs and R388::IS26 (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 IS26 from Tn4352B (pRMH975) to determine whether both tnp26 from Tn4352B were expressed equally. There was no significant difference to the right-hand IS26 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 IS26 constructs in different contexts

| Plasmid   | Structure | Mean transposition frequency (range) | tnp26 expression |
|-----------|-----------|--------------------------------------|------------------|
| pRMH973   | ![Diagram](pRMH973) | $3.1 \times 10^{-4}$ (1.9 $\times 10^{-4}$–5.1 $\times 10^{-4}$) | 1                |
| pRMH974   | ![Diagram](pRMH974) | $3.2 \times 10^{-4}$ (1.9 $\times 10^{-4}$–4.1 $\times 10^{-4}$) | 0.91 (0.57–1.29) |
| pRMH975   | ![Diagram](pRMH975) | $4.9 \times 10^{-4}$ (3.5 $\times 10^{-4}$–7.1 $\times 10^{-4}$) | 0.87 (0.77–1.05) |
| pRMH977   | ![Diagram](pRMH977) | $2.1 \times 10^{-4}$ (1.9 $\times 10^{-4}$–2.4 $\times 10^{-4}$) | 1.09 (0.64–1.40) |
| pRMH979   | ![Diagram](pRMH979) | $3.4 \times 10^{-4}$ (2.9 $\times 10^{-4}$–3.8 $\times 10^{-4}$) | 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::IS26 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 Harmar et al. (14).

IS26 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 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::Tn4352B and R388::Tn4352 were tested. Excision of the TU from R388::Tn4352B 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::Tn4352 was not detected, even after five cycles of growth without selection (Table 3).

The large A/C2 plasmid pRMH760, from which the Tn4352B-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 adaB 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 Tn4352 (Table 3). Loss of neomycin resistance was also assessed using cells carrying pDG0100, the A/C2 plasmid carrying Tn4352 in merA of pDU. No neomycin-susceptible colonies were detected after five cycles of growth of pDG0100-containing cells without selection (Table 3), consistent with the results obtained when Tn4352 was cloned into pUC19.

Exclusion of a plasmid from R388::pUC19 cointegrates. The potential product of excision from cointegrates formed between R388::IS26 (Tpr) and pRMH973 (Apr) or pRMH974 (Apr) carry-
ing a single IS26 (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 Apr Tps 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 Tn4352. 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 Tps 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 IS26-bounded structures without the additional G nucleotides, but at very low frequency.

### DISCUSSION

IS26 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 IS26 movement is conservative (14). It involves the pairing of preexisting copies of IS26 and can account for the formation of regions containing multiple copies of IS26 interspersed with the same or different DNA segments. In these structures, compound transposons share an IS26. In the current study, we have confirmed experimentally that the reverse of this conservative reaction rarely occurs by showing that the IS26-bounded transposon Tn4352 is very stable. This would ensure the stable maintenance of IS26-bounded transposons. This observation is consistent with the previously reported requirement for homologous recombination to resolve cointegrates formed by IS26 (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 IS26 and the

### TABLE 2 Testing TU excision from Tn4352B derivatives with inactive Tnp26

| Plasmid | Structure | % of Km r cells ±
|---------|-----------|----------------
|         |           | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 |
| pRMH761 |           |       |       |       |       |       |
| pRMH990 |           |       |       |       |       |       |
| pRMH994 |           |       |       |       |       |       |
| pRMH995 |           |       |       |       |       |       |

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

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

| Plasmid | Transposon | Expt ± | % of Km r cells after the following cycle (no. of generations): |
|---------|------------|--------|---------------------------------------------------------------|
|         |            | 1 (22) | 2 (44) | 3 (66) | 4 (88) | 5 (110) |
| R388::Tn4352B | Tn4352B | 1      | 83     | 46     | 23     | 15    | 7     |
|            |          | 2      | 88     | 54     | 30     | 15    | 12    |
|            |          | 3      | 92     | 58     | 36     | 18    | 8     |
| R388::Tn4352 | Tn4352  | 1–3    | 100    | 100    | 100    | 100   |
| pRMH760   | Tn4352B | 1      | 100    | 84     | 53     | 21    | 5     |
|           |          | 2      | 100    | 87     | 52     | 24    | 7     |
|           |          | 3      | 100    | 86     | 49     | 24    | 6     |
| pDGO100  | Tn4352   | 1–3    | 100    | 100    | 100    | 100   |

* Three independent experiments were performed.
* 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::IS26 cointegrate | % of Ap° Tp° coloniesa |
|-------------------------|------------------------|
| 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° colonies from each cycle.

aphA1a segment can be formed from Tn4352B 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 IS26 must encode an active transposase for integration to occur at high frequency (14), only an active left-hand IS26 (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 IS26 is likely to be involved in the excision event. The position of the extra G nucleotides, directly adjacent to this end in Tn4352B, 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, IS26-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 IS26 family, including IS257 and IS2126 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 ΔlacU169 [Δ60 lacZΔM15] hisD17 recA1 endA1 gyrA96 thi-1 relA1) was used to propagate plasmids. E. coli UB1637 (lys hist trp lac rec A 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 as an endogenous control (primers RH1466 and RH1467). Real-time PCR was performed in triplicate on independent biological replicate samples.

Plasmids. The plasmids used in this study are listed in Table 6. pRMH760 (19) and pDG0100 (18) are naturally occurring A/C, plasmids that carry Tn4352B and Tn4352, respectively. R388 is an IncW plasmid that conjugates at high frequency, and R388::IS26 was described previously (14). pRMH761 (Apr Kmr Nmr) has been described previously (14, 17) and consists of a 8.8-kb BamHI fragment from pRMH760 (bases 11784 to 12667 in GenBank accession number K976462) cloned into pUC19 (Apr). The BamHI fragment contains Tn4352B (Kmr Nmr) in the trn gene of a class 1 integron. pRMH762 is a kanamycin-sensitive derivative of pRMH761 containing only a single IS26 and was described previously (14). Plasmid pRMH991 was generated from pDG0100 (18), which contains Tn4352 in merA of pDU. Tn4352 was amplified together with 566 bp of merA on the left and 1,000 bp on the right using primers RH1462 and RH1463, 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 Tn4352 in the pRMH761 genetic context (mutations 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 pRMH976. The reciprocal construct (pRMH992), with Tn4352B in the pDG0100 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 tsp26 of Tn4352B and Tn4352 was generated as follows. Five micrograms of purified pRMH761 plasmid DNA was digested with 10 U of BsiWI for 2 h at 37°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 both IS26 and bases 52 to 55 relative to the first base of the tsp26 gene. For pRMH990, this was confirmed by sequencing the left and right IS26 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 tsp26 gene leading to premature termination at a stop codon (bases 154 to 156 in IS26), forming only a 30-amino-acid (aa) peptide.

To generate transposons with a frameshift in only the left or right tsp26 of Tn4352B, pRMH761 and pRMH990 were digested with 10 U of
Swal. The 1.8-kb fragment internal to Tn4352B 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 IS26 from pRMH761. The presence of the mutation was confirmed by sequencing both ISs in each construct.

Plasmids containing a single IS26 were constructed via PCR amplification followed by cloning into pUC19 such that the 5′ end of tsp26 was closest to P(lac). The right-hand IS26 from pRMH761 (Tn4352B) and pRMH976 (Tn4352) was amplified using primers RH1454 and RH1455 and the 9.6-kb fragment of the other generated pRMH994 with a frameshift in the right-hand IS26 tsp26. The presence of the mutation was confirmed by sequencing both ISs in each construct.

Plasmids used in this study

| Plasmid           | Description                                      | Transposon | Resistance phenotype | Reference |
|-------------------|--------------------------------------------------|------------|----------------------|-----------|
| pRMH760          | A/C2 plasmid containing Tn4352B in trnA           | Tn4352B    | Ap Cm Gm Km Km Su Tb Tp | 19        |
| pRMH761          | 8.8-kb BamHI fragment of pRMH760 containing      | Tn4352B    | Ap Km Nn             | 14        |
| pRMH976          | pRMH761 derivative containing Tn4352 in trnA     | Tn4352     | Ap Km Nn             | This study|
| pRMH990          | pRMH761 derivative with frameshifts in both tsp26| Tn4352     | Ap Km Nn             | This study|
| pRMH994          | pRMH761 derivative with a frameshift in the right tsp26 | Tn4352 | Ap Km Nn             | This study|
| pRMH995          | pRMH761 derivative with a frameshift in the left tsp26 | Tn4352 | Ap Km Nn             | This study|
| pDGO100          | pRMH761 plasmid containing Tn4352 in pDU mera    | Tn4352     | Ap Km Nn             | 18        |
| pRMH991          | 4.3-kb fragment of pDGO100 containing Tn4352 in pDU mera cloned into pUC19<sup>a</sup> | Tn4352 | Ap Km Nn | This study |
| pRMH992          | pRMH991 derivative containing Tn4352B in pDU mera<sup>a</sup> | Tn4352B    | Ap Km Nn             | This study|
| R388::IS26       | R388 with IS26                                   | Tn4352B    | Km Nn Su Tp          | 14        |
| R388::Tn4352B    | R388 containing Tn4352B<sup>b</sup>              | Tn4352B    | Km Nn Su Tp          | 14        |
| pRMH973          | Right IS26 from pRMH761 cloned into pUC19<sup>c</sup> | Tn4352 | Ap | This study |
| pRMH974          | Right IS26 from pRMH761 cloned into pUC19<sup>c</sup> | Tn4352 | Ap | This study |
| pRMH975          | Left IS26 from pRMH761 cloned into pUC19<sup>c</sup> | Tn4352 | Ap | This study |
| pRMH977          | pRMH762 derivative<sup>e</sup>                   | Tn4352B    | Ap                    | 14        |
| pRMH979          | pRMH762 derivative<sup>e</sup>                   | Tn4352B    | Ap                    | 14        |

<sup>a</sup> Ap, ampicillin; Cm, chloramphenicol; Gm, gentamicin; Km, kanamycin; Nm, neomycin; Su, sulfamethoxazole; Tb, tobramycin; Tp, trimethoprim.
<sup>b</sup> 1.8-kb central Swal Tn4352B fragment replaced with Tn4352 from pGO100B.
<sup>c</sup> Frameshift generated by end filling the BsiWI site and duplicating 116 to 119 bp from the left end of IS26 as shown in Fig. 5 of Harmer et al. (14).
<sup>d</sup> Tn4352B from pGO100B cloned into pUC19 in the same orientation as pRMH761, with the 5′ end of tsp26 closest to P(lac).
<sup>e</sup> Bases 75 to 4320 from GenBank accession number KT207465.
<sup>f</sup> Tn4352B from pGO100B cloned into pUC19 in the same orientation as pRMH761, with the 5′ end of tsp26 closest to P(lac).
<sup>g</sup> Frameshift generated by end filling the BsiWI site and duplicating 116 to 119 bp from the left end of IS26 as shown in Fig. 5 of Harmer et al. (14).
<sup>h</sup> Tn4352B from pGO100B cloned into pUC19 in the same orientation as pRMH761, with the 5′ end of tsp26 closest to P(lac).
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