Conjugative Interaction Induces Transposition of IS\textit{Pst}9 in \textit{Pseudomonas stutzeri} AN10\textsuperscript{v}

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\textit{ISPst9} is an ISL\textit{3}-like insertion sequence (IS) that was recently described in the naphthalene-degrading organism \textit{Pseudomonas stutzeri} strain AN10. In this paper we describe a novel strong IS regulation stimulus; transposition of \textit{ISPst9} is induced in all \textit{P. stutzeri} AN10 cells after conjugative interaction with \textit{Escherichia coli}. Thus, we observed that in all \textit{P. stutzeri} AN10 cells that received genetic material by conjugation the \textit{ISPst9} genomic dose and/or distribution was changed. Furthermore, \textit{ISPst9} transposition was also observed when \textit{P. stutzeri} AN10 cells were put in contact with the plasmidless conjugative strain \textit{E. coli} S17-1\textsubscript{pir}, but not when they were put in contact with \textit{E. coli} DH5\textsubscript{a} (a nonconjugative strain). The mechanism of \textit{ISPst9} transposition was analyzed, and transposition was shown to proceed by excision from the donor DNA using a conservative mechanism, which generated 3- to 10-bp deletions of the flanking DNA. Our results indicate that \textit{ISPst9} transposes, forming double-stranded DNA circular intermediates consisting of the IS and a 5-bp intervening DNA sequence probably derived from the \textit{ISPst9} flanking regions. The kinetics of IS circle formation are also described.

Insertion sequences (ISs) are mobile genetic elements that can transpose within the host genome. Most of these movements generate detrimental mutations. Because of this, ISs and their hosts have coevolved so that transposition is downregulated by many different mechanisms (for a review, see reference 28). Nevertheless, the frequency of IS transposition can increase when the cell is under stress in order to increase the chance of beneficial mutations to overcome detrimental conditions. Many physical and chemical stresses, such as starvation, high temperature, the presence of magnetic fields, shortwave irradiation, lack of oxygen availability, and metal ion exposure, have increased transcription of \textit{inhA} (transposase-encoding gene) or IS transposition frequencies in different hosts that have been studied (6, 10, 13, 14, 35, 37). Independent of the source of stress used, the IS transposition frequencies obtained in all these studies were relatively low. Furthermore, none of the stimuli used activated transposition in all of the cells influenced by the stimuli.

Many transposition mechanisms have been described, although they can be placed in two groups, replicative transposition (16) and conservative or “cut-and-paste” transposition (36), depending on whether an IS copy is conserved or not conserved in its original localization, respectively. A “cut-and-paste” transposition implies that there is excision of the IS from the original position or donor DNA. This excision can be precise (only the IS is excised) or imprecise (a few extra base pairs are excised together with the IS). It has been shown that imprecise excision can contribute to gene variation (34). Both precise and imprecise “cut-and-paste” transposition events result in changes in the position of the IS in the genome. But the mobile element can also be lost if the IS is not inserted after it is excised from a replicon. An increase in the IS copy number can also be caused by a conservative mechanism if transposition takes place between two sister chromosomes after genome duplication but prior to cell division (2).

\textit{ISPst9} is an IS belonging to the ISL\textit{3} family recently found in the naphthalene-degrading organism \textit{Pseudomonas stutzeri} strain AN10 and its 4-chlorosalicylate-degrading derivative AN142 (8). This IS is a 2,472-bp element that is flanked by two perfect 24-bp inverse repeats (IRs), which generates 8-bp AT-rich target duplication upon insertion. \textit{ISPst9} was found to transpose in multiple copies, and this IS was responsible for the \textit{nahH} (catechol 2,3-dioxygenase-encoding gene) insertional inactivation observed in strain AN142 (8). When selective pressure was applied, this IS was excised precisely to generate \textit{NahH\textsuperscript{+}} revertants that expressed the required active catechol 2,3-dioxygenase (8).

In this paper we describe a novel stimulus that is able to induce \textit{ISPst9} transposition; conjugative interaction seems to activate \textit{ISPst9} transposition in all cells that receive the stimulus. The mechanism and kinetics of \textit{ISPst9} transposition are also described.

MATERIALS AND METHODS

\textbf{Bacterial strains, plasmids, media, and culture conditions.} \textit{P. stutzeri} AN10 is a naphthalene-degrading strain that was isolated from West Mediterranean marine sediments (3, 4, 30). Routine strain confirmation analysis was performed by using 16S rRNA gene and the ITS1 sequence as previously described (17). \textit{Escherichia coli} DH5\textsubscript{a} (19) was used to maintain plasmids pBluescript SK (Stratagene) and pCR2.1 (Invitrogen) and their derivatives. \textit{E. coli} S17-1\textsubscript{pir} (20) was used as the donor strain for conjugation and for maintenance of plasmids pUT mini-Tn5-Km (9), pDSK519 (24), pGP704 (26), and pKNG101 (23) and their derivatives. \textit{E. coli} and \textit{P. stutzeri} strains were grown at 30°C in Luria-Bertani (LB) medium (31) and mineral basal medium (MBM) (1) supplemented with 0.5% (wt/vol) succinate, respectively. When appropriate, ampicillin (Ap) (100

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µg/ml), kanamycin (Km) (50 µg/ml), streptomycin (Sm) (50 µg/ml), and trimethoprim (Tp) (50 µg/ml) were added to the media.

Standard DNA manipulation. Standard DNA procedures were used in this study (31). Genomic DNA was prepared as described previously (11). Plasmid DNA was isolated by alkaline lysis using a QIAPrep Spin miniprep kit (Qiagen). Restriction endonuclease digestion (Promega and GE Healthcare) and ligation with T4 DNA ligase (Invitrogen) were performed as recommended by the manufacturer.

Hybridization and gene probes. Southern blot hybridization was performed as described previously (31). Enhanced chemiluminescence direct labeling (ECL manufacturers.

Transformation, conjugation, and cell-cell interaction. Natural transformation was performed by using the plate transformation procedure (33), with slight modifications. Briefly, an aliquot of a stationary-phase P. stutzeri culture was spotted onto a membrane filter (nitrocellulose; 0.22 µm; Millipore). When the dot containing cells was dried (after approximately 15 min at room temperature), 50 µl of EColR-linearized plasmid DNA (300 ng/µl) was added to it. The filter with the DNA-cell mixture was incubated at 30°C for 24 h on the surface of an LB agar plate.

Conjugative plasmids were transferred from E. coli S17-1αpir into P. stutzeri by filter mating. Aliquots of late-exponential-phase cultures (A600 = 0.8) of donor and recipient strains were spotted together onto a membrane filter (nitrocellulose; 0.22 µm; Millipore). When the dot containing cells was dried (after approximately 15 min at room temperature), 50 µl of EColR-linearized plasmid DNA (300 ng/µl) was added to it. The filter with the DNA-cell mixture was incubated at 30°C for 24 h on the surface of an LB agar plate.

After incubation, cell mixtures from both horizontal gene transfer events (transformation and conjugation) were resuspended in Ringer's solution (Merck). For transconjugant-transformant isolation, cell suspensions and serial dilutions of the suspensions were plated in duplicate on MM MB agar plates containing succinate plus the appropriate antibiotic.

Contact event experiments with P. stutzeri and E. coli (strain DH5α or S17-1αpir, lacking a plasmid) were performed as the conjugation experiments, except for the cell ratio used. In order to ensure that there was interaction with P. stutzeri, in the absence of a detectable genetic marker for selection, up to 10 to 10 6 E. coli cells per cell of P. stutzeri were used. Briefly, 10-fold serial dilutions (10 1 to 10 6) of a late-exponential-phase culture of P. stutzeri (1 to 10 9 CFU/ml) were spotted together with an aliquot of a late-exponential-phase culture of E. coli (10 9 CFU/ml) onto membrane filters (nitrocellulose; 0.22 µm; Millipore).

After incubation at 30°C for 7 h on the surface of LB agar plates, cell mixtures were resuspended in Ringer's solution and directly plated onto MM MB agar plates containing succinate as a unique carbon and energy source for P. stutzeri containing 

RESULTS AND DISCUSSION

First evidence of induced ISp9 transposition. Since it has been demonstrated previously that ISp9 can transpose in multiple copies at a reasonably high frequency (up to 10 3) (8), we tried to increase the native ISp9 transposition frequency to facilitate study of the transposition mechanism. Therefore, harvested P. stutzeri AN10 stationary-phase cells were subjected to different stresses, including high salinity (2 h of incubation at 30°C with up to 27% [wt/vol] NaCl), acidic and basic pHs (2 h of incubation at 30°C at pH 5.6 and 11.6, respectively), an elevated temperature (2 h of incubation at 47°C), and a high level of UV light radiation (45 min of exposure at 20°C to 300 J/m²). In spite of the low level of survival obtained in each case (the survival frequencies were between 10 4 and 10 0), no modification of the ISp9 pattern was observed for the P. stutzeri AN10 survivors analyzed after genomic DNA digestion, followed by Southern blot hybridization with the tspA4 (ISp9 transposase-encoding gene) probe. An increase in ISp9 transposition was detected in an experiment to generate mutants with mutations in the nahAc (naphthalene 1,2-dioxygenase) and nahG (salicylate 1,2-hydroxylase) genes of P. stutzeri AN10. Two plasmids, pLAN04 and pLAN05, containing internal fragments of the nahAc and nahG genes, respectively, both derived from a lacZ-Km cage, were constructed to introduce by conjugation and double homologous recombination lacZ-Km translational fusions into both genes (M. P. Lanfranconi, unpublished). As both of these plasmids are derivatives of suicide vectors (pLAN04 is a derivative of pKNG101, and pLAN05 is a derivative of pGSP074) that are stable only in λpir derivatives of E. coli, they were not able to replicate in P. stutzeri AN10. Both plasmids were transferred from E. coli S17-1αpir into P. stutzeri AN10, and Km-resistant transconjugants were selected. The resulting Km colonies were expected to be transconjugants that had incorporated the lacZ-Km cassette by homologous recombination between the flanking nah regions in the plasmid and the native nah gene in the recipient cells. Transconjugants in which double homologous recombination had occurred were selected as Km-resistant Sm-sensitive colonies and Km-resistant Ap-sensitive colonies for pLAN04 and pLAN05, respectively. Eight of
the transconjugants (four mutants with mutations in nahG and four mutants with mutations in nahAc) were selected, and the fidelity of recombination was checked by Southern blot hybridization. Thus, EcoRI-digested genomic DNAs from transconjugants were hybridized with internal nahG and nahAc probes.

The hybridization patterns expected using both internal gene probes were not observed. Further analysis involving hybridization of the same Southern blot with *tnpA4* produced two unexpected results (Fig. 1): (i) each of the nahAc and nahG mutants analyzed had a unique *tnpA4* hybridization pattern that was totally different from the pattern observed for the wild-type strain, showing that there was an increased number of putative IS*Pst9* copies; and (ii) the culture of *P. stutzeri* AN10 used to obtain both mutants had an extra copy of IS*Pst9* probe revealed the same *tnpA4* probe. As observed with the recombination-dependent DNA hybridization bands (as shown in Fig. 1) which might have occurred due to the presence of a mixture of clones with different IS*Pst9* genomic distributions. As expected, Southern blot hybridization of the EcoRI-digested genomic DNAs of the selected transconjugants with the *tnpA4* probe revealed that IS*Pst9* transposition occurred in all of the AN10 derivatives analyzed; in 44% of both types of mutants (nahAc and nahG) there was an increase in IS*Pst9* copy number, in around 40% of both types of mutants two copies were maintained, although there were changes in their locations, and in only 15% of both types of mutants one or both copies of IS*Pst9* were lost (Table 1).

Conjugation experiments with *E. coli* S17-1*λ*par carrying pUT mini-Tn5-Km (9) and *P. stutzeri* AN10 were also performed. The aim of this analysis was to evaluate whether another system of foreign DNA maintenance that disturbed the structure of the chromosome (transposition of mini-Tn5-Km) also up-regulated IS*Pst9* transposition. Twenty-eight transconjugants in which a *tnpA4* distribution was checked by hybridization with the *tnpA4* probe. As observed with the recombination-dependent DNA acquisition, for all clones that acquired the Km determinant occurrence, for all clones that acquired the Km determinant transposition in transconjugants, ad-

As both foreign DNA chromosomal integration methods resulted in 100% IS*Pst9* transposition in transconjugants, additional experiments were performed in order to evaluate whether acquisition of self-replicative DNA by conjugation without foreign DNA integration could also induce IS*Pst9* transposition. Conjugation experiments with *E. coli* S17-1*λ*par.
carrying the broad-host-range plasmid pDSK519 (24) and *P. stutzeri* AN10 were carried out. Thirty transconjugants harboring the pDSK519 plasmid were selected as Km-resistant clones and were checked to determine whether there were changes in the ISₚₛ₉₉ genomic distribution. For 28 clones (93.3%) that acquired the pDSK519 plasmid by conjugation there were changes in the original tnpA₄ hybridization pattern (Table 1 and Fig. 2A), suggesting that the increase in ISₚₛ₉₉ transposition was caused by the addition by conjugation of foreign DNA, independent of the mechanism of maintenance or other events in the host.

Transformation assays were also performed with plasmid pDSK519 and *P. stutzeri* AN10. These experiments were carried out to clarify whether there were horizontal gene transfer mechanisms other than conjugation that were able to upregulate ISₚₛ₉₉ transposition. Twenty-three transformants were se-

<table>
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<tr>
<th>Plasmid</th>
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<th>Transconjugants analyzed</th>
<th>ISₚₛ₉₉ changesᵃ</th>
<th>No. of ISₚₛ₉₉ copies</th>
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<tr>
<td>pLAN04</td>
<td>Homologous recombination in nahAc gene</td>
<td>27</td>
<td>27 (100)</td>
<td>12 (44)</td>
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<tr>
<td>pLAN05</td>
<td>Homologous recombination in nahG gene</td>
<td>27</td>
<td>27 (100)</td>
<td>12 (44)</td>
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<tr>
<td>pUT mini-Tn5-Km</td>
<td>Transposition</td>
<td>28</td>
<td>28 (100)</td>
<td>10 (36)</td>
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<tr>
<td>pDSK519</td>
<td>Plasmid replication</td>
<td>30</td>
<td>28 (93)</td>
<td>8 (27)</td>
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ᵃ Increase, presence of more than two copies of ISₚₛ₉₉; Pattern, two copies of ISₚₛ₉₉, but with a different hybridization pattern; Loss, presence of less than two copies. The values are the number (percentages) of transconjugants in each group.
ᵇ Number (percentage) of transconjugants that had a different ISₚₛ₉₉ genomic distribution than the wild-type strain.

FIG. 2. Southern blot hybridization with the tnpA₄ probe for ISₚₛ₉₉ (A) and the tnpA₂ probe (B) of EcoRI-digested genomic DNAs from *P. stutzeri* AN10 (lane WT) and 30 derivatives that received plasmid pDSK519 by conjugation. Triangles indicate EcoRI DNA fragments that hybridized with the wild-type strain with both probes used. Dots indicate tnpA₄-containing EcoRI DNA fragments of transconjugants that lost ISₚₛ₉₉ after conjugation. Relationships between labeled bands are indicated by colors (black and white). The star indicates a tnpA₂-containing EcoRI DNA fragment of transconjugants whose presence cannot be directly related to ISₚₛ₉₉ transposition, as explained in the text. Arrows indicate the absence of bands that revealed plausible genome rearrangements produced during ISₚₛ₉₉ transposition, as explained in the text.
lected as Km-resistant clones. As expected (25), Southern blot hybridization with pDSK519 revealed that the linear plasmid introduced into strain AN10 by natural transformation did not recirculate to reestablish its replicative conformation but was inserted randomly into the genome of all transformants analyzed (results not shown). Although the random insertion altered the genome structure of transformants, none of them showed variation in the tnpA4 hybridization pattern. This suggested that ISPst9 transposition was enhanced only by conjugative DNA acquisition.

On other hand, all Southern blot membranes containing EcoRI-digested DNAs of the 112 AN10 transconjugants analyzed previously (Table 1) were also hybridized with a probe for tnpA2, the plausible transposase gene in an IS5-like insertion sequence located next to nahW (salicylate 1,2-dihydroxylase-encoding gene) (5) and close to ISPst9. The aim of these hybridization experiments was to analyze whether there was a similar effect on the transposition of other IS elements present in P. stutzeri AN10. The wild-type strain exhibited hybridization signals with 10 distinct EcoRI-EcoRI DNA fragments (Fig. 2B), suggesting that at least 10 additional tnpA2-like genes were present. In fact, the existence of three such genes has been reported previously; one of them is next to the naphthalene degradation upper pathway genes (tnpA1) (3), and the other two flank the nahW gene (tnpA2 and tnpA3, located in the same EcoRI-EcoRI DNA fragment) (5). As shown for the transconjugants that received pDSK519 (Fig. 2B), only two tnpA2 hybridization bands of P. stutzeri AN10 were affected. Interestingly, both bands were the same size as the tnpA4 hybridization bands observed for the wild-type strain (Fig. 2A).

Moreover, in all cases in which a transconjugant did not contain the 7.2-kb EcoRI-EcoRI ISPst9-containing DNA fragment as determined by hybridization, the corresponding tnpA2-containing band was also not present. In almost all these cases, a new tnpA2-containing band was detected (Fig. 2B, lane 30). This new tnpA2-containing band was at 4.7 kb, suggesting that there had been an ISPst9 excision event. Similar behavior was observed for the larger ISPst9-containing band (Fig. 2A). When this IS copy disappeared from its original location, the corresponding tnpA2-containing band (Fig. 2B) also disappeared, and a new smaller 2.5-kb tnpA2-containing band appeared (Fig. 2B, lane 30). Interestingly, a new 13-kb EcoRI-EcoRI tnpA2-containing band was detected in some of the transconjugants (Fig. 2B, lane 30). As far as we could tell, this new tnpA2-containing band was not directly correlated with any observed ISPst9 hybridization pattern, and it was also found in some transconjugants (Fig. 2B, lanes 3 and 14) that did not show changes in the ISPst9 genomic distribution. In any case, as this new tnpA2-containing band always appeared at the same position, independent of the clone analyzed, we assumed that its appearance was not due to IS5-like random transposition and could have been due to other ISPst9 transposition events that have not been detected by hybridization experiments yet. Finally, some transconjugants (Fig. 2B, lane 1) that had lost the ISPst9 copy on the 7.2-kb EcoRI-EcoRI DNA fragment did not contain the tnpA2-containing 4.7-kb EcoRI-EcoRI DNA fragment mentioned above (Fig. 2B). This could be attributed to genome rearrangements resulting from ISPst9 transposition that led to deletion or a change in position of the flanking DNA fragments. Similarly, genome rearrangements caused by ISPpu12, one of the closest relatives of ISPst9, have been reported previously (38). Thus, we concluded that conjugation only upregulates ISPst9 transposition and does not stimulate IS5-like transposition.

Additional experiments were performed in order to determine whether the upregulation of ISPst9 transposition in P. stutzeri AN10 was due to the conjugative interaction with E. coli or whether the presence of E. coli during the mating event alone was enough to activate ISPst9. Thus, separate contact experiments with strain AN10 and two E. coli K-12 derivatives, one having the conjugation machinery (strain S17-1-1λpu) and the other not having the conjugation machinery (strain DH5α), were performed. None of the E. coli strains carried plasmids. Because no genetic exchange was intended to occur between either of the E. coli strains and AN10, there was no marker for selecting AN10 cells which came into contact with E. coli. Therefore, to force interactions between cells of the different species, strain AN10 was serially diluted, and 1 to 100 cells of this strain were spotted together with 10^6 cells of E. coli. Although there was no absolute guarantee of interaction, up to 6 of the 16 AN10 isolates obtained which had been in contact with E. coli S17-1-1λpu showed ISPst9 transposition (Fig. 3A). On the other hand, no transposition of this IS was observed in the 24 isolates of P. stutzeri AN10 which had been in contact with E. coli DH5α (Fig. 3B).

Simon and coworkers reported (32) that during construction of E. coli S17-1 (the parental strain of E. coli S17-1-1λpu), in a conjugation experiment the chromosomally integrated RP4 plasmid responsible for the conjugative process could be precisely excised and transferred to the receptor strain at low frequencies. We evaluated this possibility by mating 10^8 AN10 cells with 10^6 cells of S17-1-1λpu. As previously reported (32), we monitored transfer of the Tp resistance determinant (harbored in the chromosomally integrated RP4 plasmid) to strain AN10 by plating preparations in MBM supplemented with Tp. The Tp' acquisition frequencies (10^-5) were lower than the acquisition frequencies obtained by Simon and coworkers for E. coli SM10 (10^-5), a close relative of S17-1. Furthermore, we analyzed the presence of the Tp' determinant in the 16 AN10 isolates obtained previously after contact with strain S17-1-1λpu (the isolates shown in Fig. 3A). All of these isolates, including the six isolates that showed ISPst9 transposition (Fig. 3A), were Tp sensitive. We also hybridized the Southern blot membrane shown in Fig. 3A with the RP4-oriT probe and obtained no signal (results not shown), which indicated that plasmid RP4 was not present in genomes of the AN10 derivatives. Thus, although we cannot be completely sure that no genetic material was transferred during the conjugative process, our results suggest that conjugative interaction causes upregulation of ISPst9 transposition. To our knowledge, no similar phenomenon has been described previously. The most similar phenomenon was described by Godoy and Fox (15), who observed a high level of Tn10 loss after conjugational transfer, which they attributed to recombination when this element was inserted by conjugation.

**ISPst9 transposed by excision from the donor DNA.** Most of transconjugants analyzed showed a unique and discriminative tnpA4 hybridization pattern, independent of their experimental origin, and seven was the highest number of copies detected (Fig. 2A, lane 21). Despite this, loss of ISPst9 copies and
position changes were also observed, as mentioned above (Fig. 2A and Table 1). However, when the total number of putative ISPst9 copies was calculated for all transconjugants used in each experimental approach (Table 1), 2.1 ± 0.2 ISPst9 copies per transconjugant were obtained, suggesting that there was a "cut-and-paste" transposition mechanism (36). PCR amplification using primers SAL64 and SAL71 (Fig. 4A) was performed to determine whether ISPst9 really moved from its original position and originated the movements and losses observed. Wild-type P. stutzeri AN10 and the 30 AN10 transconjugants harboring plasmid pDSK519 shown in Fig. 2 were used. As expected, a single approximately 3.3-kb PCR product was obtained for the wild-type strain and all of the transconjugants in which ISPst9 was on the 7.2-kb EcoRI-EcoRI DNA fragment (Fig. 4B, lane 29). On the other hand, all of the pDSK519-containing transconjugants that had lost ISPst9 at the original position and produced the 4.7-kb tnpA2-containing DNA band (Fig. 2B) produced a unique PCR product that was approximately 0.85 kb long (Fig. 4B, lane 30). This result suggested that there was excision of ISPst9 from the donor DNA, supporting the hypothesis that there were genome rearrangements during ISPst9 transposition, as mentioned above.

The first evidence of ISPst9 transposition was observed with P. stutzeri AN142 (8). In this strain, a copy of ISPst9 disrupted the nahH gene. This copy of ISPst9 was flanked by perfect 8-bp direct repeats that were generated at the moment of insertion, and it could be excised precisely in order to reestablish functionality of the nahH gene. This was not observed for the original copy of ISPst9 in P. stutzeri AN10, for which no 8-bp direct repeats were found (8). In order to evaluate whether the excision of ISPst9 occurred together with deletion of a fixed number of nucleotides (the expected 8 bp), six of the 0.85-kb PCR products obtained from transconjugants which had lost the copy of ISPst9 indicated in Fig. 4A were sequenced. Nucleotide sequencing confirmed that ISPst9 was excised nearly perfectly, although the amount of the flanking DNA deleted during transposition ranged from 3 to 10 bp (Fig. 4C). Previous reports indicated that there are more perfect mobile element excision events than imperfect mobile element excision events. This probably occurs because a reestablished phenotype is necessary for detection of IS excision, since regeneration in frame of the inactivated gene is the only way that this can occur. In our experiment there was no phenotypic selection,
and thus the deletion of a fixed number of nucleotides (8 bp) was not essential, although perfect excision could also occur, as previously demonstrated (8).

**ISP**<sub>9</sub> transposition forming IS circle intermediates. **IS**<sub>1411</sub> (22), another member of the **IS**<sub>L3</sub> family, is one of the many **IS**s that have been shown to transpose via **IS** circle formation (7). To prove that circle formation also occurred in **IS**<sub>Pst9</sub>, a PCR was performed using primers **ISMG2** and **ISMG4**, which hybridized at the ends of **IS**<sub>Pst9</sub> with an outward orientation (Fig. 4A). Thus, if **IS**<sub>Pst9</sub> circle formation occurred, a 0.95-kb PCR product was expected. Genomic and plasmid DNA extraction protocols were used to obtain DNA from contact between **P. stutzeri** AN10 and **E. coli** S17-1/H9261<sub>pir</sub> and from both strains separately as controls. An intense PCR product of the expected size was obtained from the contact event, independent of the DNA extraction protocol used, whereas only a very faint band was obtained when the DNA came from **P. stutzeri** AN10 alone (Fig. 5A). No amplification was observed with **E. coli** S17-1/H9261<sub>pir</sub> (Fig. 5A). **ISP**<sub>9</sub> circle formation was also analyzed by PCR amplification for contact between strain AN10 and **E. coli** DH5α. Only a faint band, similar to the band obtained for strain AN10 alone, was observed. Therefore, we suggest that basal **ISP**<sub>9</sub> circle formation occurs in **P. stutzeri** AN10 and is enhanced during conjugative interaction. More precisely, as circles were detected after alkaline lysis-based DNA extraction, we also suggest that the **IS** circles were double-stranded DNA elements. Moreover, **IS** circle PCR amplification was also obtained after digestion with mung bean nuclease (Fig. 5B). No amplification was observed after thermal DNA denaturation prior to nuclease digestion, confirming the degrading effect of mung bean nuclease on single-stranded DNA.

The amplified 0.95-kb PCR product was sequenced using primers **ISMG2** and **ISMG4**. As expected, the nucleotide sequence confirmed that the two IRs of **ISP**<sub>9</sub> were contiguous and were separated by an imperfect 5-bp sequence (Fig. 5C).
This 5-bp sequence was probably a combination of the two IS\textit{Pst}9 flanking sequences. A similar result was obtained for IS\textit{1411}, although the 5-bp sequence (5′-AAACC-3′) that separated the IRs was derived from the left IR flanking sequence (22).

In order to evaluate the kinetics of IS\textit{Pst}9 circle formation, contact event experiments with \textit{P. stutzeri} AN10 and \textit{E. coli} S17-1\textit{λ}\textsubscript{pir} were performed using different incubation times (1 to 24 h). PCR amplification with ISMG2 and ISMG4 revealed that maximal IS\textit{Pst}9 circle formation occurred within the first 3 h of contact (Fig. 5D). Three independent experiments were done in order to analyze the kinetics of IS\textit{Pst}9 circle formation during the first 4 h of contact. The results demonstrated that maximum circle formation occurred after 2.5 h, although an increase in IS\textit{Pst}9 circle formation compared to the AN10 basal activity was observed after only 45 min of conjugative interaction (Fig. 5D). It has been shown previously that the transposase of IS\textit{911}, one of the best-studied IS, is able to generate IS circles after 16 min of induction (12). However, these elevated IS\textit{911} circle formation kinetics were obtained by
cloning the transposase gene under transcriptional control of the P_tac promoter and not under natural conditions used here.

The results obtained for ISPtr9 transposition are the first results for self-inducible, real, in vivo kinetics without any artificial transposase transcriptional amplification. This sensitive, easy transposition detection measurement method, together with the fact that conjugating interacting P. stutzeri cells mobilize ISPtr9, should allow workers to study in depth the signaling cascade that occurs in the host after the stimulus that upregulates transposition, as well as the consequences of transposition for the host.

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