Conjugative Interaction Induces Transposition of IS*Pst9* in *Pseudomonas stutzeri* AN10[∇]

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IS*Pst9* is an IS*L3*-like insertion sequence (IS) that was recently described in the naphthalene-degrading organism *Pseudomonas stutzeri* strain AN10. In this paper we describe a novel strong IS regulation stimulus; transposition of IS*Pst9* is induced in all *P. stutzeri* AN10 cells after conjugative interaction with *Escherichia coli*. Thus, we observed that in all *P. stutzeri* AN10 cells that received genetic material by conjugation the IS*Pst9* genomic dose and/or distribution was changed. Furthermore, IS*Pst9* transposition was also observed when *P. stutzeri* AN10 cells were put in contact with the plasmidless conjugative strain *E. coli* S17-1λ$_{pir}$, but not when they were put in contact with *E. coli* DH5α (a nonconjugative strain). The mechanism of IS*Pst9* 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 IS*Pst9* transposes, forming double-stranded DNA circular intermediates consisting of the IS and a 5-bp intervening DNA sequence probably derived from the IS*Pst9* 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 *tnpA* (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 re-

sult 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).

IS*Pst9* is an IS belonging to the IS*L3* family recently found in the naphthalene-degrading organism *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. IS*Pst9* was found to transpose in multiple copies, and this IS was responsible for the *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 NahH$^+$ revertants that expressed the required active catechol 2,3-dioxygenase (8).

In this paper we describe a novel stimulus that is able to induce IS*Pst9* transposition; conjugative interaction seems to activate IS*Pst9* transposition in all cells that receive the stimulus. The mechanism and kinetics of IS*Pst9* transposition are also described.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and culture conditions. *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). *Escherichia coli* DH5α (19) was used to maintain plasmids pBluescript SK (Stratagene) and pCR2.1 (Invitrogen) and their derivatives. *E. coli* S17-1λ$_{pir}$ (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. *E. coli* and *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 manufacturers.

Hybridization and gene probes. Southern blot hybridization was performed as described previously (31). Enhanced chemiluminescence direct labeling (ECL direct nucleic acid labeling and detection system; GE Healthcare) was used for hybridization. Gene probes were prepared by PCR amplification using *Taq* DNA polymerase (GE Healthcare) and *P. stutzeri* AN10 or *E. coli* S17-1$_{pir}$ genomic DNA as the template. The templates and primers used to obtain the probes were as follows: for the 0.64-kb *tnpA4* (IS*Pst9* transposase-encoding gene) probe, template strain AN10 and primers ISMG3 and ISMG9 (8); for the 0.46-kb *tnpA2* (IS*5*-like transposase-encoding gene) probe, template strain AN10 and primers IS5-1F (5′-ACYTTCGCCGAYGCCGAGTA-3′) and IS5-1R (5′-GCCRTCCT TGTTCTTGGTCG-3′); and for the 1.03-kb RP4-oriT (origin of transfer of plasmid RP4 and flanking regions) probe, template strain S17-1$_{pir}$ and primers oriT-F (5′-GGCTTGGCCTTGATGTGCCG-3′) and oriT-R (5′-CGTGCTTG GCAATCACGCGC-3′). In all cases, the PCR cycling conditions were as follows: 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min and then 72°C for 10 min.

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 dry (after approximately 15 min at room temperature), 50 μl of EcoRI-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* S17-1$_{pir}$ into *P. stutzeri* by filter mating. Aliquots of late-exponential-phase cultures (A_{600}, ∼0.8) of donor and recipient strains were spotted together onto a membrane filter (nitrocellulose; 0.22 μm; Millipore) to obtain a cell ratio of 1:1. The mixture was incubated at 30°C for 7 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 onto MBM agar plates containing succinate plus the appropriate antibiotic.

Contact event experiments with *P. stutzeri* and *E. coli* (strain DH5α or S17-1$_{pir}$ lacking a plasmid) were performed like 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 10^7 to 10^8 *E. coli* cells per cell of *P. stutzeri* were used. Briefly, 10-fold serial dilutions (10^{-6} to 10^{-8}) of a late-exponential-phase culture of *P. stutzeri* (1 to 100 CFU) were spotted together with an aliquot of a late-exponential-phase culture of *E. coli* (10^8 CFU) 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 MBM agar plates containing succinate as a unique carbon and energy source for *P. stutzeri* counting. Serial dilutions of cell mixtures were plated onto LB agar plates for total (*P. stutzeri* and *E. coli*) cell counting. For further genomic analysis only *P. stutzeri* cells from contact events in which the *P. stutzeri*/*E. coli* ratio was between 1:10^7 and 1:10^8 were used.

IS*Pst9* transposition analysis. The location of IS*Pst9* in the *nah* cluster of *P. stutzeri* AN10 and its derivatives was routinely analyzed by PCR amplification with *Taq* DNA polymerase (GE Healthcare), using primers SAL64 and SAL71 as previously described (8).

In order to evaluate the kinetics of IS*Pst9* transposition, contact event experiments with *E. coli* S17-1$_{pir}$ lacking a plasmid and *P. stutzeri* were performed as described above but with different incubation times. After contact, cell mixtures were resuspended in 500 μl of Tris-EDTA buffer (50 mM Tris-HCl, 20 mM sodium EDTA; pH 8.0), and genomic and plasmid DNAs were extracted as described above. The quality and concentration of the extracted DNAs were assessed with a NanoDrop ND-1000 UV-visible spectrophotometer (NanoDrop Technologies) used according to the manufacturer's instructions. IS*Pst9* circle formation was detected by performing PCR with 12 ng/μl (final concentration) of extracted DNA, 0.3 μM outward primer ISMG2 (5′-GCTCACGAAGTCTCA GACC-3′), and 0.3 μM outward primer ISMG4 (5′-TGGTCAGTGCACCTCG TTC-3′). The PCR cycling conditions were as follows: 94°C for 5 min, followed

by 25 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min and then 72°C for 10 min. IS*Pst9* circle formation was considered to occur when a unique 0.95-kb PCR product was observed. After three independent experiments, the PCR products of each set of experiments were resolved on a 1% agarose gel and stained with ethidium bromide. PCR products were semiquantified using the GeneTools version 3.04.04 analysis program (SynGene). For analysis, the highest measured signal was defined as 100%.

Restriction with mung bean nuclease (Invitrogen) was performed to evaluate the DNA structure (single-stranded DNA or double-stranded DNA) of IS*Pst9* circle intermediates. Briefly, a 50-μl aliquot containing 1 μg of genomic DNA extracted after a mating event was treated with 10 U of mung bean nuclease for 30 min at 30°C. A control for enzyme functionality was included, using DNA that was thermally denatured (5 min at 100°C) and rapid chilling on ice). After treatment, samples were purified by phenol-chloroform-isoamyl alcohol (25:24:1) extraction and ethanol precipitation. Each pellet was resuspended in 30 μl of sterile deionized water for further IS circle amplification by PCR as described above.

Sequencing. For sequencing, PCR products were purified with a QIAquick PCR purification kit (Qiagen). Sequences were determined a using BigDye Terminator cycle sequencing v3.1 kit (Applied Biosystems) according to the manufacturer's instructions. Sequence analyses were performed using the BioEdit 6.0.5 sequence alignment editor (18).

RESULTS AND DISCUSSION

First evidence of induced IS*Pst9* transposition. Since it has been demonstrated previously that IS*Pst9* can transpose in multiple copies at a reasonably high frequency (up to 10^{-5}) (8), we tried to increase the native IS*Pst9* 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/m^2). In spite of the low level of survival obtained in each case (the survival frequencies were between 10^{-4} and 10^{-6}), no modification of the IS*Pst9* pattern was observed for the *P. stutzeri* AN10 survivors analyzed after genomic DNA digestion, followed by Southern blot hybridization with the *tnpA4* (IS*Pst9* transposase-encoding gene) probe.

An increase in IS*Pst9* 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 disrupted by a *lacZ*-Km^r cassette, were constructed to introduce by conjugation and double homologous recombination *lacZ*-Km^r 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 pGP704) that are stable only in λ$_{pir}$ derivatives of *E. coli*, they were not able to replicate in *P. stutzeri* AN10. Both plasmids were transferred from *E. coli* S17-1$_{pir}$ into *P. stutzeri* AN10, and Km^r transconjugants were selected. The resulting Km^r colonies were expected to be transconjugants that had incorporated the ′*lacZ*-Km^r 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
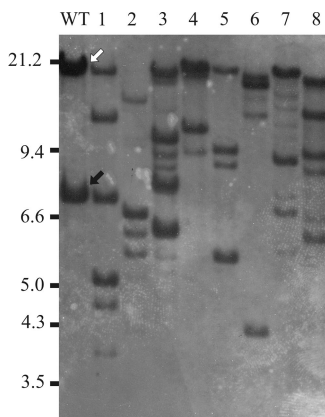
FIG. 1. Southern blot hybridization with the *tnpA4* probe of EcoRI-digested genomic DNAs from *P. stutzeri* AN10 (lane WT) and mutant derivatives with mutations in the *nahAc* (lanes 1 to 4) and *nahG* (lanes 5 to 8) genes. Previously described (8) and newly described IS*Pst9*-containing EcoRI-EcoRI bands are indicated by black and white arrows, respectively.

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 *tnpA4* on a 21-kb EcoRI-EcoRI DNA fragment (Fig. 1) compared to the clone in which IS*Pst9* was found previously, which had a unique copy of *tnpA4* in a 7.2-kb fragment (Fig. 1) (8).

The clone of *P. stutzeri* strain AN10 used for generation of mutants was recovered in this study from a long-term culture frozen at −80°C, whereas the first analysis described (8) was performed with a culture which had been passed an unknown number of times on LB agar plates with long intervals of plate preservation at 4°C. In order to clarify the difference in the number of *tnpA4* copies in the genome of *P. stutzeri* AN10, up to eight more isolates were obtained from other stored cultures, including the originally deposited AN10 clone from the CCUG culture collection (CCUG 29243) at the University of Göteborg. The identity of these isolates as *P. stutzeri* strain AN10 was confirmed by 16S rRNA gene and ITS1 sequence analysis (17). Southern blot hybridization of their EcoRI-digested genomic DNAs with the *tnpA4* probe revealed the same hybridization pattern in each case: two bands at 7.2 and 21 kb (as shown for the wild type in Fig. 1). PCR amplification using primers SAL64 and SAL71, which hybridize with flanking DNA of the originally described IS*Pst9* copy (8), was also carried out to demonstrate that this copy remained in its original location. As expected, a 3.32-kb PCR product was obtained. Thus, as all of the results showed unequivocally that in

P. stutzeri AN10 there are copies of *tnpA4* in two different EcoRI-EcoRI DNA fragments and as both copies were shown to be mobile (Fig. 1), we suggest that they are in two independent mobile elements. In this scenario, we attribute the single copy of IS*Pst9* found in the strain used in previous work (8) to copy loss that occurred during long-term plate storage. This is not the first time that transposition of mobile elements induced by starvation conditions has been described. Some examples of this behavior are IS*H27* of *Halobacterium halobium* (29), IS*5* and IS*30* of *E. coli* (27), and Tn*4652* of *Pseudomonas putida* (21), showing that ISs can be important in bacterial variation under laboratory conditions.

IS*Pst9* transposition was induced by conjugative interaction. A series of conjugation experiments with *E. coli* S17-1λ*_{pir}* carrying pLAN04 or pLAN05 and *P. stutzeri* AN10 were performed in order to quantify the observed upregulation of IS*Pst9* transposition. Fifty-four transconjugants in which a double homologous recombination event had occurred were selected (27 *nahAc*::*lacZ*-Km^r, Km-resistant, Sm-sensitive transconjugants and 27 *nahG*::*lacZ*-Km^r, Km-resistant, Ap-sensitive transconjugants). Before genomic DNA was extracted, at least two passages on LB medium supplemented with kanamycin were performed using single colonies. This strategy was routinely used in order to avoid the presence of low-intensity *tnpA4* 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* S17-1λ*_{pir}* carrying pUT mini-Tn*5*-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-Tn*5*-Km) also upregulated IS*Pst9* transposition. Twenty-eight transconjugants in which transposition of mini-Tn*5*-Km occurred were selected as Km-resistant Ap-sensitive clones, and their IS*Pst9* genomic distribution was checked by hybridization with the *tnpA4* probe. As observed with the recombination-dependent DNA acquisition, for all clones that acquired the Km^r determinant by conjugation followed by transposition there were changes in the *tnpA4* hybridization pattern (Table 1). The number of clones that showed an increase in the IS*Pst9* copy number was slightly reduced compared with the values obtained in recombination-dependent DNA acquisition experiments. Interestingly, higher percentages of clones that had lost IS*Pst9* copies or in which the location was changed were observed (Table 1).

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* S17-1λ*_{pir}*

TABLE 1. IS*Pst9* transposition data obtained with different transconjugants of *P. stutzeri* AN10

Plasmid	Event after conjugation	Transconjugants analyzed		IS*Pst9* changes[a]			No. of IS*Pst9* copies	
		n	IS*Pst9* affected[b]	Increase	Pattern	Loss	Total	Mean
pLAN04	Homologous recombination in *nahAc* gene	27	27 (100)	12 (44)	11 (41)	4 (15)	55	2.04
pLAN05	Homologous recombination in *nahG* gene	27	27 (100)	12 (44)	10 (37)	5 (9)	69	2.55
pUT mini-Tn5-Km	Transposition	28	28 (100)	10 (36)	5 (18)	13 (46)	57	2.04
pDSK519	Plasmid replication	30	28 (93)	8 (27)	1 (3)	19 (63)	56	1.87

[a] Increase, presence of more than two copies of IS*Pst9*; Pattern, two copies of IS*Pst9*, 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 IS*Pst9* genomic distribution than the wild-type strain.

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 IS*Pst9* 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 IS*Pst9* transposi-

tion 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 IS*Pst9* transposition. Twenty-three transformants were se-
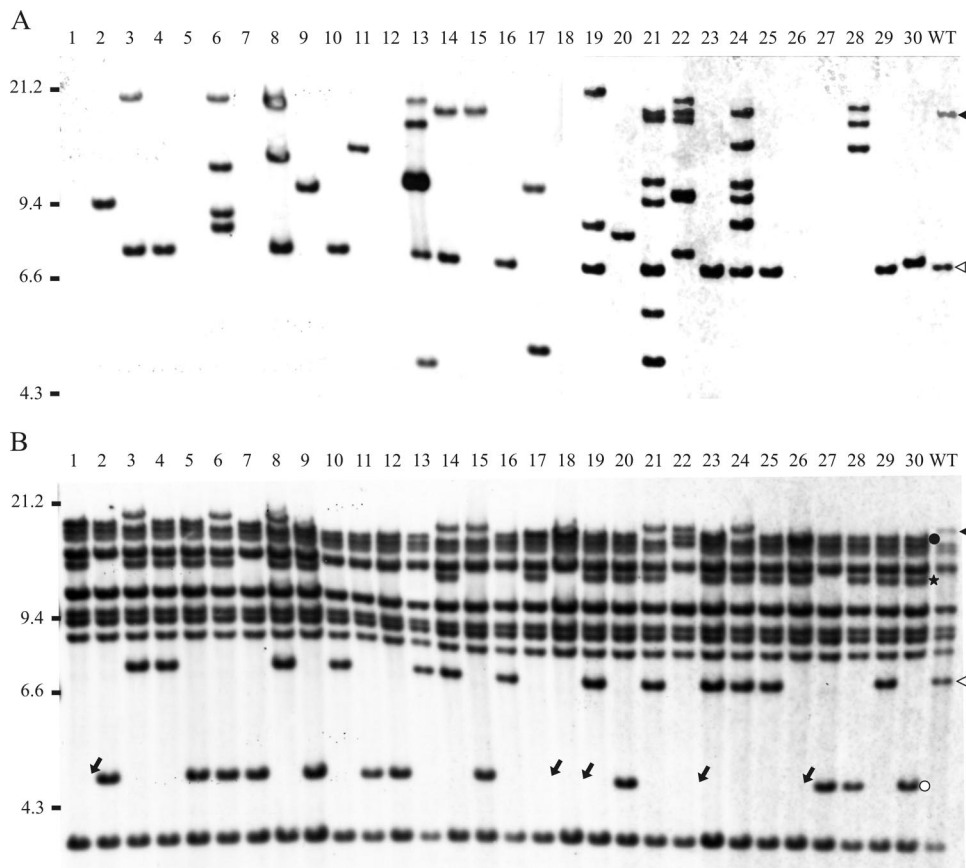

FIG. 2. Southern blot hybridization with the *tnpA4* probe for IS*Pst9* (A) and the *tnpA2* probe (B) of EcoRI-digested genomic DNAs from *P. stutzeri* AN10 (lane WT) and 30 derivatives that received plasmid pDSK519 by conjugation. Triangles indicate EcoRI DNA fragments that hybridized with the wild-type strain with both probes used. Dots indicate *tnpA2*-containing EcoRI DNA fragments of transconjugants that lost IS*Pst9* 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 IS*Pst9* transposition, as explained in the text. Arrows indicate the absence of bands that revealed plausible genome rearrangements produced during IS*Pst9* 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-hydroxylase-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 event experiments with strain AN10 and two *E. coli* K-12 derivatives, one having the conjugation machinery (strain S17-$1\lambda_{pir}$) 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 10^8 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-$1\lambda_{pir}$ 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-$1\lambda_{pir}$), 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 10^8 cells of S17-$1\lambda_{pir}$. 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^r acquisition frequencies (10^{-8}) 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^r determinant in the 16 AN10 isolates obtained previously after contact with strain S17-$1\lambda_{pir}$ (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 Tn*10* 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
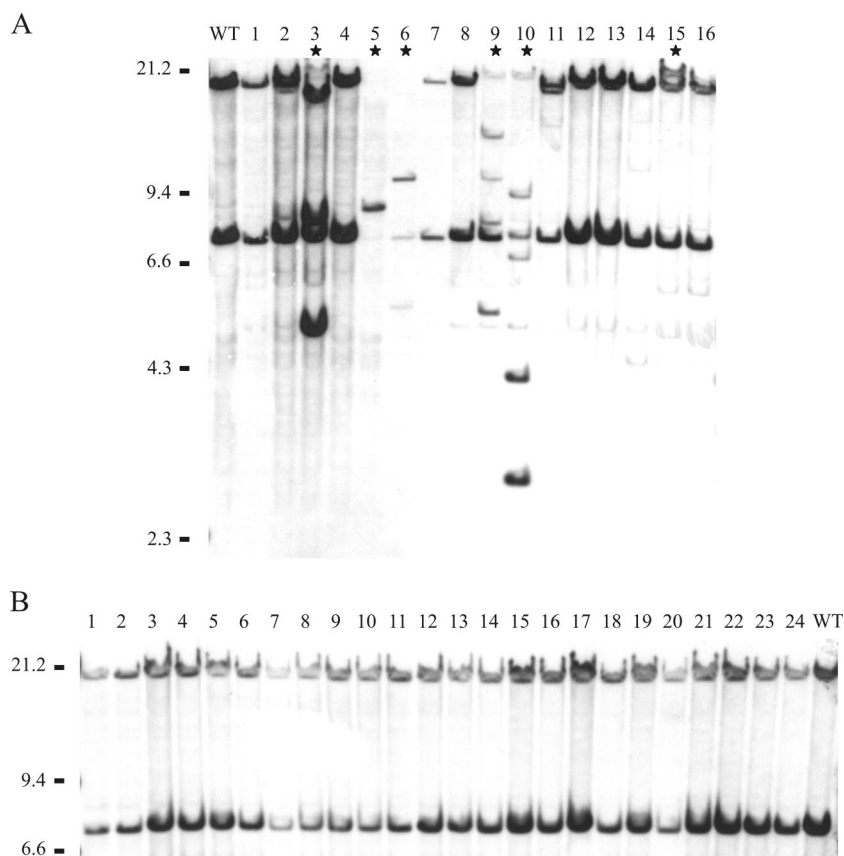
FIG. 3. Southern blot hybridization with the *tnpA4* probe of EcoRI-digested genomic DNAs from *P. stutzeri* AN10 and derivatives isolated after contact with *E. coli* S17-1λ*pir* (A) or *E. coli* DH5α (B). Lane WT contained *P. stutzeri* wild-type strain AN10. Stars indicate the isolates showing IS*Pst9* transposition.

position changes were also observed, as mentioned above (Fig. 2A and Table 1). However, when the total number of putative IS*Pst9* copies was calculated for all transconjugants used in each experimental approach (Table 1), 2.1 ± 0.2 IS*Pst9* 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 IS*Pst9* 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 IS*Pst9* 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 IS*Pst9* 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 IS*Pst9* from the donor DNA, supporting the finding that this IS transposed using a nonreplicative mechanism. Interestingly, no amplification was obtained for four of the five transconjugants (Fig. 4B) that had lost both the *tnpA4* and *tnpA2* hybridization bands mentioned above (Fig. 2B). This could have been due to plausible deletion of IS*Pst9*-flanking DNA after transposition. The

fifth transconjugant (Fig. 2B, lane 22) produced a PCR product whose size was identical to the size of the product observed for the wild-type strain (Fig. 4B). This result supports the hypothesis that there were genome rearrangements during IS*Pst9* transposition, as mentioned above.

The first evidence of IS*Pst9* transposition was observed with *P. stutzeri* AN142 (8). In this strain, a copy of IS*Pst9* disrupted the *nahH* gene. This copy of IS*Pst9* 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 IS*Pst9* in *P. stutzeri* AN10, for which no 8-bp direct repeats were found (8). In order to evaluate whether the excision of IS*Pst9* 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 IS*Pst9* indicated in Fig. 4A were sequenced. Nucleotide sequencing confirmed that IS*Pst9* 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,
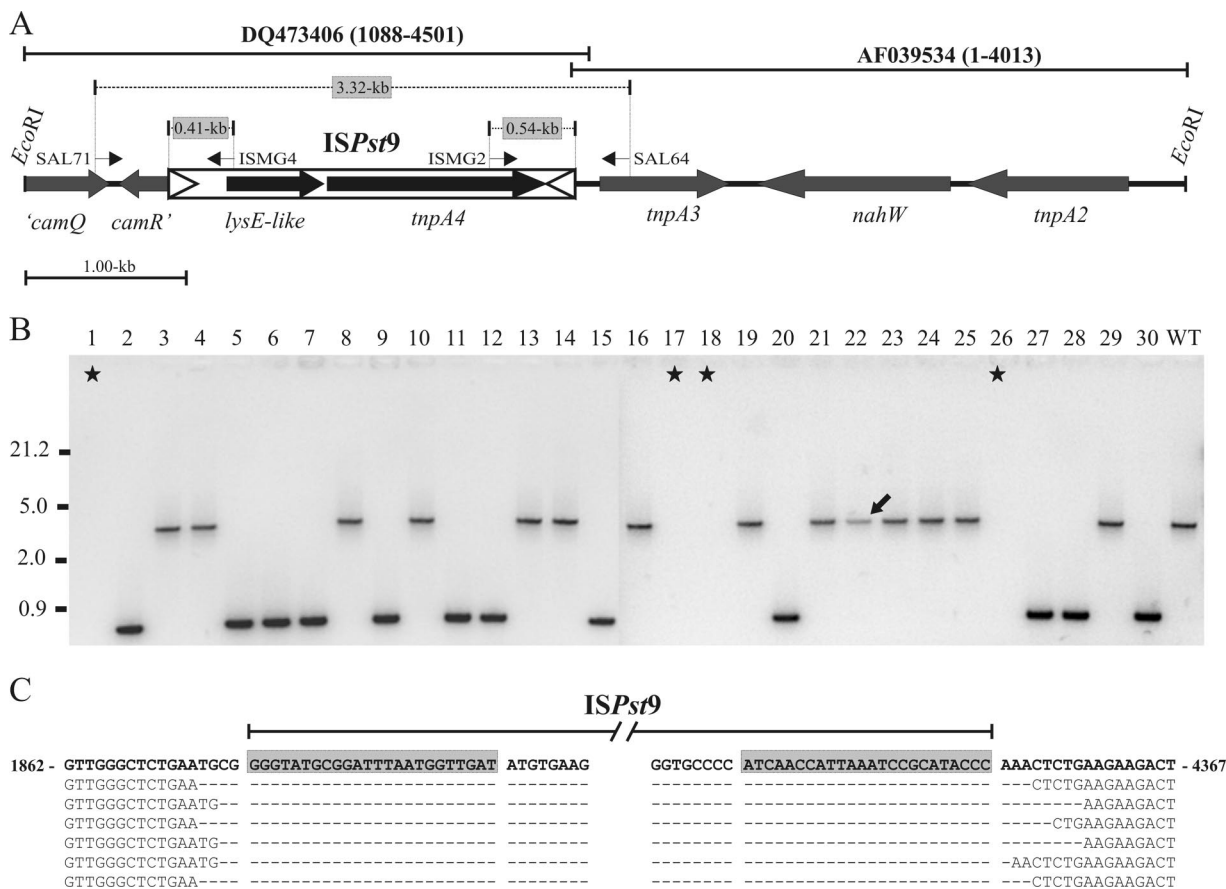
FIG. 4. Location of ISPst9 in P. stutzeri AN10. (A) Schematic diagram of the 7.2-kb EcoRI-EcoRI DNA fragment of P. stutzeri AN10 harboring ISPst9. DQ473406 and AF039534 are nucleotide database accession numbers. Coordinates of both sequences are indicated in parentheses. The entire naphthalene degradation lower pathway (nahGTHINLOMKJ) is located in a 5.8-kb EcoRI-EcoRI fragment immediately upstream from the tnpA2 gene (4, 5). Genes code for the following proteins: 'camQ, partial CamQ lactone hydrolase-like protein; camR', partial CamR transcriptional regulator; lysE-like, LysE-like protein; tnpA4, ISPst9 transposase; tnpA2 and tnpA3, putative IS5-like transposases; nahW, salicylate hydroxylase. The arrows indicate the directions of gene transcription. SAL71, ISMG4, ISMG2, and SAL64 were the primers used; the thin arrows indicate their locations and 5′-3′ orientations. Open triangles indicate IRs of ISPst9. (B) PCR amplification with primers SAL71 and SAL64 of P. stutzeri AN10 (lane WT) and the 30 derivatives that received plasmid pDSK519 by conjugation. Stars and the arrow indicate the isolates suspected to have had genome rearrangements during ISPst9 transposition. (C) Alignment of ISPst9 flanking sequences from P. stutzeri AN10 and six derivatives that received pDSK519 by conjugation and produced a single 0.85-kb SAL71-SAL64 PCR product. Nucleotides 1862 to 4367 of the previously published sequence of strain AN10 (accession number DQ473406) are indicated by bold type. IRs of ISPst9 are shaded.

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).

ISPst9 transposition forming IS circle intermediates. IS1411 (22), another member of the ISL3 family, is one of the many ISs that have been shown to transpose via IS circle formation (7). To prove that circle formation also occurred in ISPst9, a PCR was performed using primers ISMG2 and ISMG4, which hybridized at the ends of ISPst9 with an outward orientation (Fig. 4A). Thus, if ISPst9 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 S17-1λ$_{pir}$ 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 S17-1λ$_{pir}$ (Fig. 5A). ISPst9 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 ISPst9 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 ISPst9 were contiguous and were separated by an imperfect 5-bp sequence (Fig. 5C).
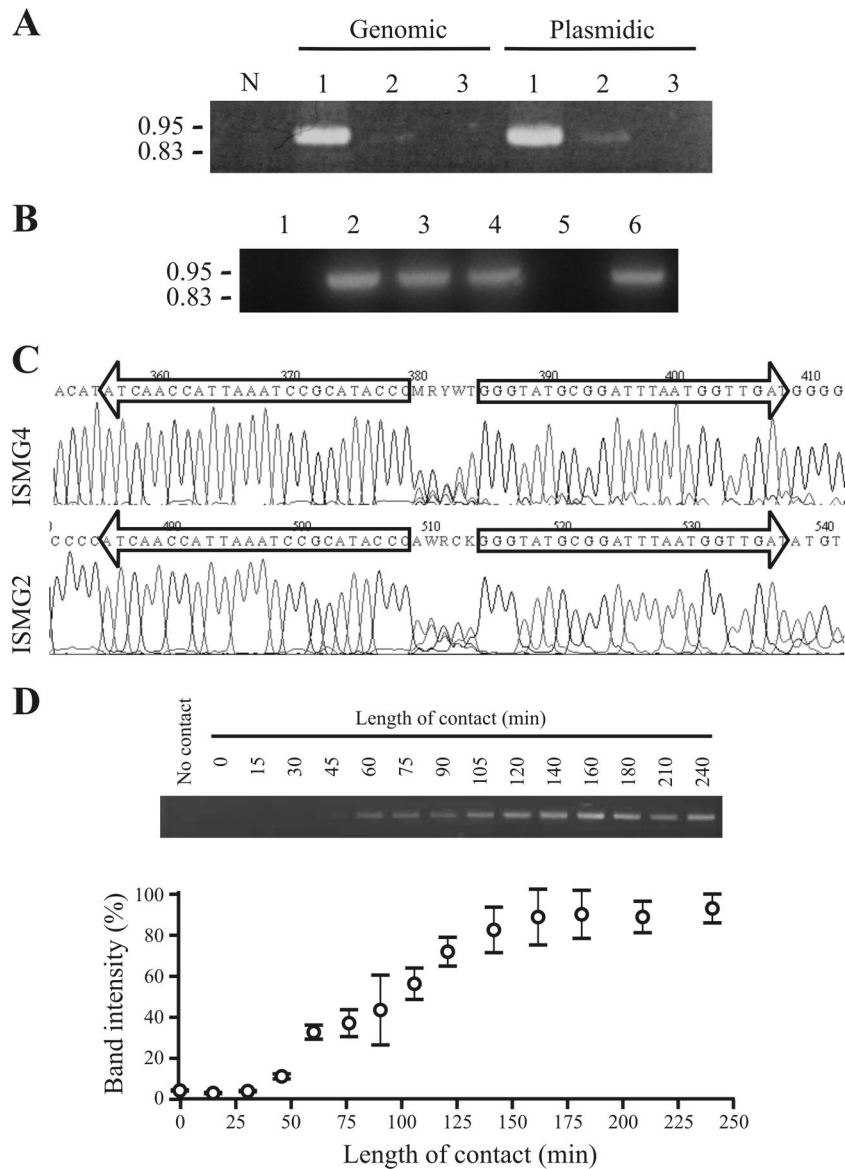
FIG. 5. Circle formation by IS*Pst9*. (A) PCR amplification of circle junctions using primers ISMG2 and ISMG4. Lane N, negative control; lane 1, contact between *P. stutzeri* AN10 and *E. coli* S17-1λ$_{pir}$; lane 2, strain AN10 alone; lane 3, strain S17-1λ$_{pir}$ alone. (B) PCR amplification of circle junctions after mung bean nuclease treatment. Lane 1, negative control; lane 2, positive control; lane 3, addition of mung bean nuclease buffer without enzyme; lane 4, treatment with mung bean nuclease; lane 5, treatment with mung bean nuclease after thermal DNA denaturation; lane 6, thermally denatured DNA without mung bean nuclease treatment. (C) Sequencing electropherograms obtained with primers ISMG2 and ISMG4 for the PCR product shown in panel A, lane 1. Only the circle junction area of chromatograms is shown. IRs of IS*Pst9* are indicated by arrows. (D) Semiquantification of IS*Pst9* circle formation in contact experiments with *P. stutzeri* AN10 and *E. coli* S17-1λ$_{pir}$ for different incubation times. Only one of the three independent gels used for semiquantification is shown. The symbols and error bars indicate the means and standard deviations of three independent experimental determinations.

This 5-bp sequence was probably a combination of the two IS*Pst9* flanking sequences. A similar result was obtained for IS*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*Pst9* circle formation, contact event experiments with *P. stutzeri* AN10 and *E. coli* S17-1λ$_{pir}$ were performed using different incubation times (1 to 24 h). PCR amplification with ISMG2 and ISMG4 revealed that maximal IS*Pst9* 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*Pst9* 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*Pst9* 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*911*, one of the best-studied IS, is able to generate IS circles after 16 min of induction (12). However, these elevated IS*911* circle formation kinetics were obtained by

cloning the transposase gene under transcriptional control of the P$_{lac}$ promoter and not under natural conditions used here.

The results obtained for IS*Pst9* 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 conjugative interacting *P. stutzeri* cells mobilize IS*Pst9*, 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.

ACKNOWLEDGMENTS

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