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TnpR Encoded by an IS*Ppu12* Isoform Regulates Transposition of Two Different IS*L3*-Like Insertion Sequences in *Pseudomonas stutzeri* after Conjugative Interaction▽†

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***Pseudomonas stutzeri* AN10 has two IS*L3*-like insertion sequences (ISs). One of them has been recently described as IS*Pst9*. In this study we show that the second IS, situated 4.5 kb upstream of IS*Pst9*, is an isoform of IS*Ppu12* from *Pseudomonas putida* mt-2. Although both IS*L3*-like ISs are flanked by nearly identical (21/24 conserved residues) inverted repeats (IRs) and harbor similar transposases (93% amino acid identity), they differ in their accompanying genes. As described for IS*Pst9*, the isoform of IS*Ppu12* also transposes by a conservative mechanism, forms circular double-stranded DNA (dsDNA) transposition intermediates, and is induced by interaction with the conjugative strain *Escherichia coli* S17-1λ_{pir} (conjugative interaction) but not with the nonconjugative *E. coli* DH5α. In fact, we demonstrate that IS*Pst9* transposition after conjugative interaction occurs only when IS*Ppu12* is present, indicating that IS*Ppu12* is upregulating transposition of both ISs under such conditions. We also demonstrate that this conjugative interaction-mediated induction of IS*Ppu12* is not exclusive to the *P. stutzeri* AN10 strain but is a more general phenomenon, at least in *Pseudomonas*. Mutation of TnpR, a MerR-like transcriptional regulator present in IS*Ppu12* but not in IS*Pst9*, reduced the transcription of *tnpA* (IS*Ppu12* transposase-encoding gene) and decreased formation of circular dsDNA transposition intermediates after conjugative interaction. Complementation of the TnpR mutant restored the phenotype. In addition, the presence of TnpR in an IS*Ppu12*-free genetic background did not induce IS*Pst9* after conjugative interaction. Thus, our results suggest that TnpR, after conjugative interaction, activates transcription of *tnpA* of IS*Ppu12*. Then, TnpA of IS*Ppu12* would bind to IRs of both ISs, IS*Ppu12* and IS*Pst9*, causing their transposition.**

Insertion sequences (ISs) are small genetic elements with the ability to transpose that are widespread in bacteria. In the genus *Pseudomonas* alone, more than 130 different ISs have been reported at the IS Finder database (http://www-is.biotoul.fr) distributed among 11 different species. So far, the ISs of *Pseudomonas* can be grouped in at least 16 different IS families based mainly on similarities in genetic organization and in the amino acid sequence of the transposases (reviewed in reference 10). Although this high dispersion of ISs in bacteria indicates a selfish effect for horizontal propagation, ISs have been described to coevolve with their hosts as mechanisms for adaptation (27, 29). Transposition in bacteria is downregulated and maintained at low levels due to its detrimental effect on cellular functions, like the ones produced in mutational processes or gene inactivation (reviewed in reference 29). Some of the regulation systems depend on the physiological state of the host cell. For example, transposition frequency increases under stress conditions, such as under starvation (22).

Pseudomonas stutzeri AN10 is a naphthalene-degrading bacterium (18) whose naphthalene catabolic genes (*nah* genes) are chromosomally encoded (33). The *nah* genes are organized in four operons (see Fig. S1 in the supplemental material): *nahABFCED* (*nah* upper pathway), coding for the enzymes involved in the conversion of naphthalene to salicylate; *nahGTHINLOMKJ* (*nah* lower pathway), coding for the conversion of salicylate to pyruvate and acetyl coenzyme A (CoA); *nahR*, the regulatory gene; and *nahW*, a second salicylate hydroxylase gene (6–8). Besides the *nah* lower pathway, a novel and functional IS element of the IS*L3* family, IS*Pst9*, was identified and characterized (11). In *P. stutzeri*, IS*Pst9* was involved in catabolic gene inactivation (interrupting gene *nahH*), like two of its closest relatives in the IS*L3* family, IS*Pst2* from *Pseudomonas* sp. OX1 (5) and IS*Ppu12* from *Pseudomonas putida* mt-2 (39). IS*Pst9* showed an apparent conservative mechanism of transposition, excising from its original position by forming circular double-stranded DNA (dsDNA) transposition intermediates (12). Hybridization of genomic DNA with a specific probe for the IS*Pst9* transposase gene (*tnpA4*) revealed the presence of a putative second IS*Pst9*-like copy in *P. stutzeri* AN10 (12). Both IS*L3*-like elements of *P. stutzeri* AN10 had the peculiarity of increasing their transposition frequency after conjugative interaction with an *Escherichia coli* pilus-producing strain (S17-1λ_{pir}), a strong IS upregulation stimulus recently reported whose signaling cascade leading to its activation remains unknown (12).

While trying to demonstrate whether the two putative IS*L3*-like elements present in *P. stutzeri* AN10 were proximate in its

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genome and constituted a composite transposon, as described in *P. putida* PP3, whose two copies of IS*Ppu12* formed a composite transposon (DEH), flanking the hydrolytic dehalogenase gene (*dehI*) and its regulator (*dehR*) (37), we discovered that the two putative IS*Pst9*-like copies were in fact different. In this work we show that the second IS*L3*-like copy in *P. stutzeri* AN10 corresponds to an IS*Ppu12* isoform. We also present results that demonstrate how the IS*Ppu12* isoform is responsible for the transposition of both IS*L3*-like ISs in *P. stutzeri* AN10 after the stimulus of conjugative interaction and show that the mobilization of both ISs is regulated by the transcriptional regulator TnpR, a *merR*-like gene product encoded in the IS*Ppu12* isoform.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and culture conditions. *P. stutzeri* strains used in this study were the following: AN10 and AN11 (18); AN10-5(pDSK519) (12); ATCC 17587, ATCC 17589, ATCC 17591, and CCUG 11256 (35); ATCC 14405, DNSP21, DSM 50227, DSM 50238, LSMN2, ST27MN3, S1MN1, and ZoBell (32); B1SMN1, B2SMN1, and ST27MN2 (33). *P. putida* mt-2 (38) and *Pseudomonas balearica* LS401 and SP1402 (3) were also used. The conjugative *E. coli* S17-1λ_{pir} strain (21), which has the *tra* functions encoded on its chromosome, was used as the donor for conjugation of broad-host-range plasmids pDSK519 (24) and pBBR1MCS-5 (25) and their derivatives and also for conjugative interaction events. The nonconjugative *E. coli* DH5α strain (20) was used for plasmid maintenance and as a negative control in conjugative interaction events. *E. coli* and *Pseudomonas* strains were grown at 30°C in Luria-Bertani medium (LB) (34) and on mineral basal medium (MBM) (2) supplemented with 0.5% (wt/vol) succinate. When appropriate, ampicillin (Ap; 100 μg/ml), kanamycin (Km; 50 μg/ml), or gentamicin (Gm; 10 μg/ml) was added to the medium. MICs for heavy metals were calculated as previously described (13), using MBM supplemented with 0.5% (wt/vol) succinate and the respective heavy metal salt [$CdCl_2$, $(CH_3COO)_2Pb \cdot 3H_2O$, $CoCl_2 \cdot 6H_2O$, $CuCl_2 \cdot 2H_2O$, $HgCl_2$, $NiCl_2 \cdot 6H_2O$, or $ZnCl_2$].

DNA manipulations and analysis. Standard DNA procedures were used throughout the study (34). Total genomic DNA preparations and Southern blot hybridizations were carried out as previously described (12). An 0.64-kb specific probe, *tnpA4*, for the transposase-encoding gene in IS*L3*-like elements was obtained using ISMG3 and ISMG9 primers (11). Specific probes for detection of IS*Pst9* and IS*Ppu12* were made by PCR as follows: 94°C for 5 min; 30 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 30 s; and 72°C for 10 min. Primers used were ISMG5-F (5′-TGAACGAGGTGCACTGACC-3′) and ISMG7-R (5′-GCGATAAAAACCG TCCCAAGAG-3′) for amplification of *lysE* (open reading frame [ORF] accompanying *tnpA4* in IS*Pst9*) and IRISL3 (5′-GGGTAWRCGGATTWAATGGTTG-3′) and ISPPU518R (5′-AGAATCCGTTGGGGAGGAGG-3′) for amplification of *tnpR* (one of the three ORFs accompanying the *tnpA* gene in IS*Ppu12*), resulting in an 0.5-kb product in both cases. Plasmid DNA was isolated by alkaline lysis using the QIAprep Spin Miniprep kit (Qiagen). Restriction endonuclease digestions (Roche and GE Healthcare) and ligations with T4 DNA ligase (Invitrogen) were done as recommended by each manufacturer. Except when specified, PCR amplifications were done using *Taq* DNA polymerase (GE Healthcare).

Conjugative interaction events to induce the mobilization of the IS*L3*-like ISs were performed with *P. stutzeri* and *E. coli* S17-1λ_{pir} lacking plasmids as previously described (12). Briefly, *P. stutzeri* and *E. coli* stationary-phase cultures were spotted together onto a membrane filter (nitrocellulose, 0.22 mm; Millipore) using a cell ratio of $1:10^6$, respectively. The mixture was incubated at 30°C for 7 h on the surface of an LB agar plate. After incubation, the cell mixture was used for cell counting and/or nucleic acid extraction. In these experiments, the nonconjugative *E. coli* DH5α strain was used as a negative control.

The analysis of IS*Pst9* circular dsDNA transposition intermediates was done by PCR with primers ISMG2 and ISMG4 as described previously (12). IS*Ppu12* circular dsDNA transposition intermediate analysis was done as for IS*Pst9* although PCR amplifications were carried out using primers ISMG2 (12) and ISPPU518R. A multiplex PCR using a 2:1:1 primer ratio of ISMG2, ISMG4, and ISPPU518R was performed in order to analyze simultaneously the formation of circular dsDNA transposition intermediates of both IS*L3*-like elements, IS*Pst9* and IS*Ppu12*. The kinetics for the formation of circular dsDNA transposition intermediates was determined with the combination of the three primers after performing conjugative interaction events with different incubation times as previously described (12). The PCR products of three independent experiments were resolved on agarose gels stained with ethidium bromide. PCR products were semiquantified using GeneTools v.3.04.04 (SynGene). The highest measured signal was defined as 100% as described previously (12).

Excision of IS*Ppu12* from its original DNA location was checked using standard PCR conditions (as above) with a 3.5-min elongation time and using primers FLANKPPU12-F (5′-ACAGGTGCAGGTCCGAACCG-3′) and FLANKPPU12-R (5′-TTGGATGCCGGCGAACACCG-3′), located at the flanking regions of the IS.

The quality and concentration of the extracted DNA were assessed with a NanoDrop ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies) according to the manufacturer's instructions. PCR bands were semiquantified using the GeneTools v.3.04.04 analysis program (SynGene).

Analysis of *tnpA* expression by RT-PCR. Conjugative interaction events were performed between *P. stutzeri* AN10 and *E. coli* S17-1λ_{pir} as described above. After 4 h of mating, total RNA was extracted with the TRIzol Max bacterial RNA isolation kit (Invitrogen) following the manufacturer's indications. DNA was digested with recombinant RNase-free DNase I (Roche) for 1 h. Complete DNA elimination was checked by performing a PCR amplification of 16S rRNA genes from the DNase-treated RNA extract with primers F27 and R1492 (26). cDNA was obtained by reverse transcription (RT) using SuperScript III reverse transcriptase (Invitrogen) and random oligonucleotides (Gibco BRL-Life Technologies) according to the manufacturer's recommendation. The final concentration of RNA used in each RT reaction was 0.5 μg/μl. Semiquantification of *tnpA* transcripts (for the transposase encoded in the IS*Ppu12* isoform) was done by RT-PCR using primers ISMG3 and ISMG9 and conditions described previously (12). Amplification of the housekeeping gene *rpoD*, using primers PsEG30F and PsEG790R and conditions described elsewhere (28), was done for signal normalization.

Plasmid construction. PCR amplification of the IS*L3*-like element intermediate region was carried out using primers ISMG2 and ISMG4 (12) at a final concentration of 0.3 μM and using cycling conditions as follows: 94°C for 5 min; 30 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 5 min; and 72°C for 10 min. The 5.0-kb amplicon obtained was cloned in pCR2.1 (TOPO-TA cloning kit; Invitrogen), producing plasmid pJOC26.

Both IS*L3*-like elements of *Pseudomonas* strains, IS*Pst9* and IS*Ppu12*, were amplified using primer IRISL3 (5′-GGGTAWRCGGATTWAATGGTTG-3′). PCR conditions were as follows: 94°C for 5 min; 30 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 3.5 min; and 72°C for 10 min. Amplifications were cloned in pCR2.1 (TOPO-TA cloning kit; Invitrogen), producing pTOPOPst9 and pTOPOPpu12. Cloned ISs were cleaved by EcoRI digestion and inserted in the unique EcoRI restriction site of the broad-host-range pBBR1MCS-5 vector (25), producing pJOC40 (with IS*Pst9*) and pJOC41 (with IS*Ppu12*).

The *tnpR*-defective IS*Ppu12* IS was obtained by amplifying plasmid pTOPO-Ppu12 by PCR using primers PPU12MF (5′-TAGTCGACCCCTACCAACTG CGCC-3′) and PPU12MR (5′-TGTTCATAGAAGCGGATCGTCGACAT-3′), both with a SalI restriction site. PCR was carried out using AccuPrime *Taq* DNA polymerase (Invitrogen), and conditions were those recommended by the manufacturer. Cycling conditions were as follows: 94°C for 30 s; 2 cycles of 94°C for 30 s, 60°C for 30 s, and 68°C for 8 min; 30 cycles of 94°C for 30 s, 68°C for 30 s, and 68°C for 8 min; and 68°C for 10 min. The amplification product was digested with SalI and autoligated, producing pTOPOPpu12mut. IS*Ppu12* with the *tnpR* mutation was cleaved from pTOPOPpu12mut by an EcoRI digestion and cloned in EcoRI-linearized pBBR1MCS-5, producing pJOC41mut. Complementation of *tnpR* was achieved by cloning the PCR amplification of a 1.7-kb fragment of IS*Ppu12* of strain AN10 (containing *tnpR*) in pCR2.1 (TOPO-TA cloning kit; Invitrogen), producing pTOPOtnpR. PCR was done using primers IRISL3 and ISPPU1696R (5′-TGCCAGCCTCCACCATTCGC-3′) and conditions as previously done for total IS amplification but limiting elongation time to only 1.5 min. The cloned fragment was excised from pTOPOtnpR by EcoRI digestion and cloned in the unique EcoRI restriction site of the broad-host-range pDSK519 vector (24), giving pJOC42.

DNA sequencing and analysis. The sequence of IS*Ppu12* of *P. stutzeri* AN10 and the intermediate region between IS*Ppu12* and IS*Pst9* was determined directly from plasmids pTOPOPpu12 and pJOC26 by using the primer walking method, with the design of new primers based on the determined sequences. To determine the DNA sequence upstream of IS*Ppu12*, an inverse PCR was carried out. For this, genomic DNA from *P. stutzeri* AN10 was digested with SmaI. The digestion was purified (PureLink PCR purification kit; Invitrogen) and autoligated. The ligation product was used as template for PCR amplification using ENT3E-F (5′-CTATGCCGGCAAGTCCATCG-3′) and ISPPU518R primers, and the amplification product was sequenced. The BigDye Terminator cycle

sequencing v3.1 kit (Applied Biosystems) was used according to the manufacturer's instructions.

Primer design and sequence analysis were done using the BioEdit 6.0.5 sequence alignment editor (19). Similarity searches with GenBank, EMBL, and PROSITE databases were done using BLASTP (NCBI), FASTA protein (EBI), and ScanProsite (SIB) web tools, respectively (1, 15, 30). IS nomenclature attribute analysis was done using BLASP and BLASTN web tools (1) at the IS Finder database (http://www-is.biotoul.fr). According to the IS Finder database, the criteria to consider a novel IS to be an isoform of a previously described IS are the following: more than 98% similarity between transposases and/or more than 95% of identity between entire IS nucleotide sequences.

Nucleotide sequence accession number. The sequence has been deposited in GenBank under accession number FJ624110.

RESULTS AND DISCUSSION

Location and identification of the second IS*Pst9*-like copy of *P. stutzeri* AN10. Weightman et al. (37) described how two copies of IS*Ppu12*, a close relative of IS*Pst9*, formed a functional composite catabolic transposon, named DEH in *P. putida* PP3. Given the facts that (i) *P. stutzeri* AN10 presented two plausible IS*Pst9* copies revealed by Southern blot hybridization (12), (ii) IS*Pst9* occurred in bacteria together with the presence of aromatic hydrocarbon-degrading determinants (11), and (iii) the IS*Pst9* copy previously localized in the chromosome of *P. stutzeri* AN10 was situated proximate to the *nah* genes (11) (see Fig. S1 in the supplemental material), we decided to investigate if the two IS*Pst9*-like copies in *P. stutzeri* AN10 constituted a composite catabolic transposon similar to DEH.

In order to evaluate if both IS*Pst9*-like copies were close together in the genome of *P. stutzeri* AN10, aliquots of genomic DNA were digested independently with different restriction enzymes. Further Southern blot and hybridization analysis with a probe for the transposase of IS*Pst9*, *tnpA4*, revealed that both IS*Pst9*-like copies coexisted in a single 15-kb StuI-StuI DNA fragment and also in a single 20-kb XhoI-XhoI DNA fragment (Fig. 1A). According to the known location of the StuI and XhoI restriction sites in the IS*Pst9* flanking sequence (7, 11), the hybridization results suggested that both IS*Pst9*-like ISs were separated by an intermediate DNA sequence of 4 to 6 kb and that the second IS*Pst9*-like element was situated upstream of the originally described copy of IS*Pst9* (11).

To amplify the intermediate region, a long elongation cycle PCR was performed using primers ISMG2 and ISMG4, which hybridized with an outward orientation at the ends of IS*Pst9* (a schematic representation of their location is shown in Fig. 2A). A PCR product of approximately 5.0 kb was obtained (Fig. 1B). After the resulting fragment was cloned in pCR2.1-TOPO (Invitrogen) to generate plasmid pJOC26, the intermediate region was sequenced, proving to be 4,479 bp in length. As described previously, sequence analysis confirmed the presence of a partial putative *camR* gene and a putative *camQ* gene (Fig. 2A) upstream of *orf1* of IS*Pst9* (11). The complete sequence of a gene (*orf2*) encoding a plausible acetoacetyl-CoA synthase (71% nucleotide identity with UPI00006D8F3B of *Pseudomonas aeruginosa*) and a putative pyruvate carboxyltransferase-encoding gene (*orf5*) (73.9% nucleotide identity to A5W2L3 of *Pseudomonas putida*) was also found (Fig. 2A). Unexpectedly, the first 481 nucleotides of the obtained sequence, which were expected to correspond to the 3′ end of IS*Pst9*, showed 100% identity with IS*Ppu12* of *P. putida* mt-2

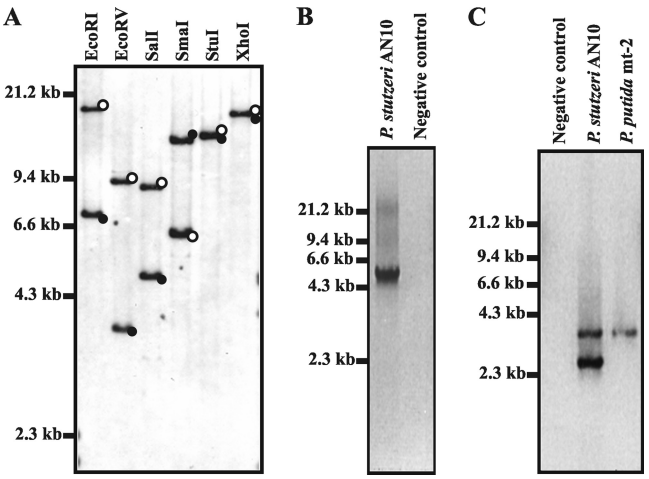

FIG. 1. (A) Southern blot hybridization with the *tnpA4* probe of *P. stutzeri* AN10 genomic DNA digested with different restriction enzymes. Black circles highlight those bands which also gave signals when hybridized with the *lysE* probe (for IS*Pst9*), whereas white circles indicate those which gave signals when hybridized with the *tnpR* probe (for IS*Ppu12*). (B) Agarose gel showing the 5.0-kb amplified product corresponding to the intermediate region between the two IS*L3*-like ISs. (C) PCR amplification obtained using primer IRISL3 when *P. stutzeri* AN10 (3.4- and 2.5-kb bands) and *P. putida* mt-2 (3.4-kb band) DNAs were used as templates.

(39) and only 92.9% identity with IS*Pst9* of *P. stutzeri* AN10 (11). This result suggested that the second IS*Pst9*-like IS present in *P. stutzeri* AN10 could be an IS*Ppu12* isoform.

In order to complete the DNA sequence of this plausible IS*Ppu12* isoform, a degenerate primer (IRISL3) was designed. The IRISL3 primer hybridized specifically with the inverted repeat (IR) sequences of IS*Pst9* and IS*Ppu12* and was used to amplify by PCR both IS*L3*-like ISs. As expected, PCR amplification using *P. stutzeri* AN10 genomic DNA as template resulted in two bands (Fig. 1C): one with the expected size for IS*Pst9* (2.5 kb) (11) and another with the expected size for IS*Ppu12* (3.4 kb) (39). Both PCR products were cloned in pCR2.1-TOPO (Invitrogen), resulting in pTOPOPst9 and pTOPOPpu12 plasmids, respectively, and both DNA inserts were sequenced. The 2,472-bp nucleotide sequence corresponding to the pTOPOPst9 insert was 100% identical to the previously sequenced IS*Pst9* of *P. stutzeri* AN10 (11). On the other hand, the analysis of the 3,372-bp nucleotide sequence of the DNA insert in pTOPOPpu12 confirmed that the second IS*Pst9*-like IS of *P. stutzeri* AN10 was an isoform of IS*Ppu12* (39) (99.9% nucleotide identity between the two entire ISs, 99.9% amino acid identity between the two transposases), according to the criteria of the IS Finder database (http://www-is.biotoul.fr). As described by Williams et al. (39) and corroborated in this study by similarity searches against GenBank, EMBL, and PROSITE databases, the IS*Ppu12* isoform of *P. stutzeri* AN10 contained four ORFs (Fig. 2B): *tnpA*, which codes for the transposase; *lspA*, encoding a plausible lipoprotein signal peptidase; *orf6*, which gave highest identities with a possible divalent heavy metal/H^+ antiporter; and *tnpR* (designated by Williams and coworkers as *orf2* [39]), the product of which seems related to *merR*-like transcriptional regulators. The previously described constitutive strong promoter P_{out}

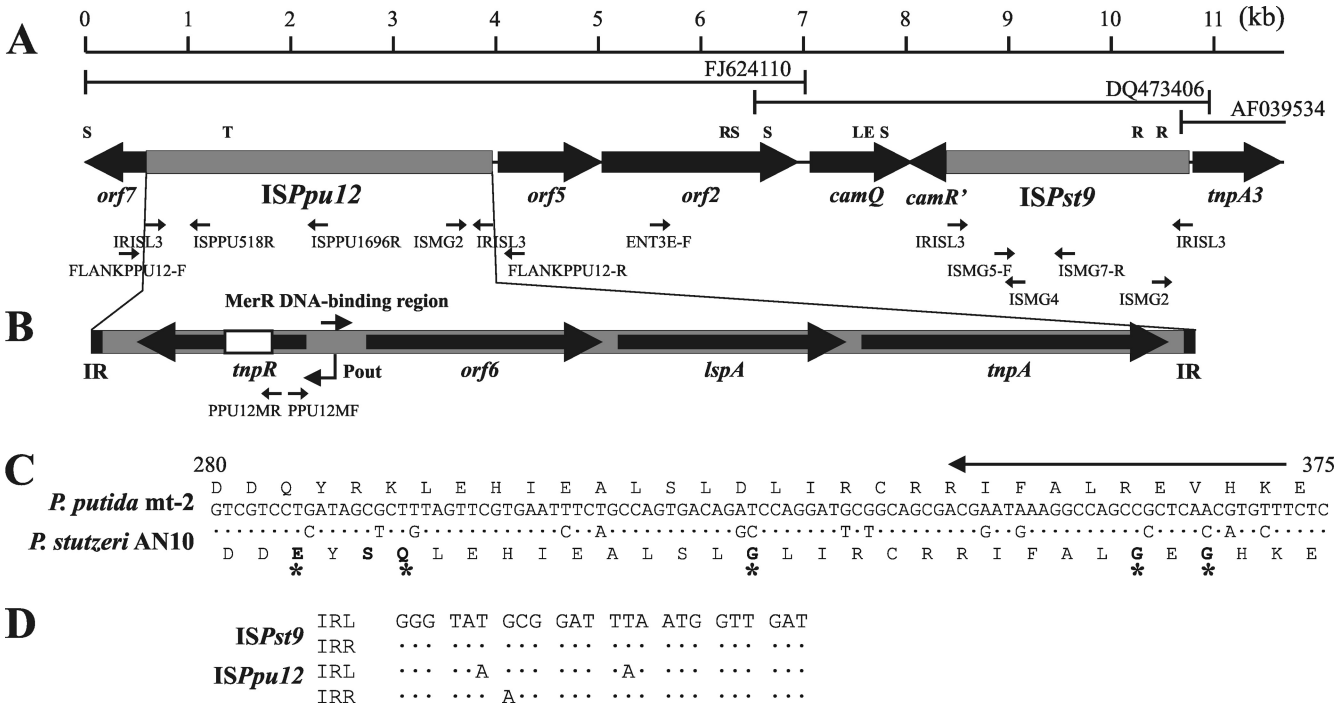

FIG. 2. (A) Schematic representation of the two IS*L3*-like ISs in *P. stutzeri* AN10 (IS*Ppu12* and IS*Pst9*). Flanking ORFs are represented with large black arrows. Sequences submitted to the databases are delimited by their accession numbers. Primers of interest and their 5′-3′ orientation are represented with small arrows. Restriction enzymes are abbreviated as follows: S, SmaI; T, StuI; R, EcoRV; L, SalI; E, EcoRI. (B) Detail of IS*Ppu12* isoform of *P. stutzeri* AN10 showing the four included ORFs and their transcription orientations. Primers used for the generation of the *tnpR* mutant are shown. The MerR-like DNA binding region and the constitutive P_{out} promoter are also represented. The white rectangle in *tnpR* indicates the variable region between IS*Ppu12* isoforms of *P. putida* mt-2 and *P. stutzeri* AN10. (C) *tnpR* variable region (280 to 375 bp) showing nucleotide and consequent amino acid differences (marked by boldface and asterisks) between the IS*Ppu12* copy of *P. putida* mt-2 and *P. stutzeri* AN10. The arrow indicates transcription orientation. (D) Left (IRL) and right (IRR) inverse repeat sequences of IS*Pst9* and IS*Ppu12*.

(Fig. 2B) (39) was also perfectly conserved. Only 18 nucleotide differences were observed between the *P. stutzeri* AN10 IS*Ppu12* isoform and IS*Ppu12* of *P. putida* mt-2; 15 of them resided in a small region of *tnpR* (Fig. 2B and C). These nucleotide differences caused 6 amino acid variations (Fig. 2C) at the end of the DNA binding domain of the plausible TnpR transcriptional regulator.

The only IS components comparable between IS*Pst9* and IS*Ppu12* isoforms of *P. stutzeri* AN10 were their transposase-encoding genes and their IR sequences. Transposases of the two ISs shared 93.0% amino acid identity, and their encoding genes showed 90.4% nucleotide identity, which explained why both ISs hybridized against the *tnpA4* probe (Fig. 1A). IS*Pst9* had two perfect 24-bp IRs, whereas IS*Ppu12* had IRs identical to the ones of IS*Pst9* except for two nucleotide differences in the left IR and one in the right one (Fig. 2D). A *tnpR* probe, specific for the IS*Ppu12* isoform, was used for hybridizing the Southern blot shown in Fig. 1A, confirming that one of the two *tnpA4*-containing bands detected for *P. stutzeri* AN10 corresponded to the IS*Ppu12* isoform (indicated with white circles in Fig. 1A).

In order to determine the nucleotide sequence upstream of the IS*Ppu12* isoform, *P. stutzeri* AN10 genomic DNA was digested with SmaI, autoligated, and used as template for PCR amplification with outward primers ISPPU518R and ENT3E-F (Fig. 2A). A 1.8-kb PCR product was obtained and sequenced. The 542 nucleotides upstream from the IS*Ppu12* isoform (Fig. 2A) revealed the presence of a partial gene, *orf7*, encoding a plausible alcohol dehydrogenase (81.0% amino acid identity with Q1LNT3 of *Ralstonia metallidurans*). No direct repeats (DRs) were flanking the IS*Ppu12* isoform in AN10, although an AT-rich sequence was observed at both sides.

IS*Ppu12* isoform transposes in *P. stutzeri* AN10 similarly to IS*Pst9*. The mobilization of IS*Pst9* in *P. stutzeri* AN10 was shown to be induced after conjugative interaction with the pilus-producing strain *E. coli* S17-1λ_{pir} (12). Therefore, in order to analyze the mechanisms of mobilization of the IS*Ppu12* isoform in *P. stutzeri* AN10, conjugation and conjugative interaction experiments were performed. In a previous experiment (12) the analysis of transposition in 30 different *P. stutzeri* AN10 derivatives that received the mobilizable plasmid pDSK519 by conjugation with *E. coli* S17-1λ_{pir} revealed that 93.3% of them (28 out 30) changed the original *tnpA4* hybridization pattern. Since our new results show that both ISs (IS*Pst9* and IS*Ppu12* isoform) were detected by the *tnpA4* probe (Fig. 1A), we analyzed if both ISs were actually moving or not. Thus, the Southern blot membranes previously hybridized against the *tnpA4* probe were separately hybridized with specific probes for the IS*Ppu12* isoform (*tnpR* probe) and for IS*Pst9* (*lysE* probe) to differentiate which bands corresponded to each one of the two IS*L3*-like elements. The two IS elements, IS*Pst9* and IS*Ppu12*, turned out to behave in a similar way after conjugative interaction, and we observed losses or increases in both IS copies, as well as changes in their position

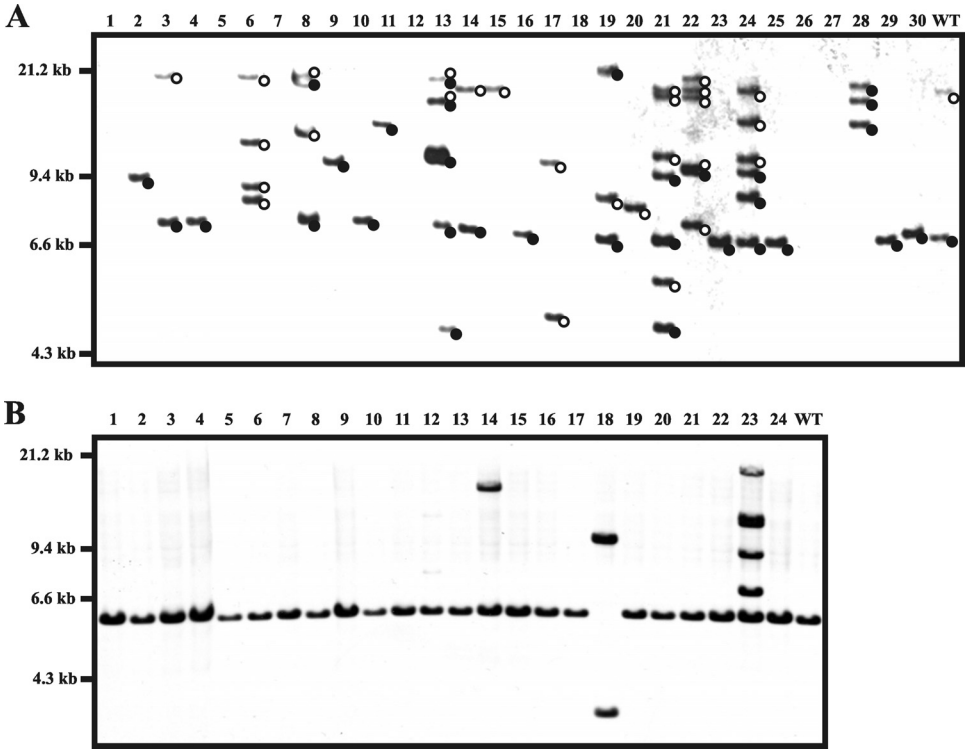

FIG. 3. (A) Southern blot assay with *P. stutzeri* AN10 (WT) and 30 different derivatives that received plasmid pDSK519 by conjugation hybridized with the *tnpA4* probe. Those bands which also gave hybridization signals with the *tnpR* probe (specific for the IS*Ppu12* isoform; white circles) and with the *lysE* probe (specific for IS*Pst9*; black circles) are indicated. (B) Southern blot hybridization with the *tnpA4* probe of EcoRI-digested genomic DNAs from *P. putida* mt-2 (WT) and 24 different derivatives which received plasmid pDSK519 by conjugation.

in the genome (Fig. 3A). The average number of each of the ISs per transconjugant was 1.02 ± 1.4 copies for the IS*Ppu12* isoform and 1.08 ± 1.2 copies for IS*Pst9*. Since the IS*Ppu12* isoform mobilized also after conjugative interaction, we used this stimulus for analyzing its mechanism of mobilization, as we did previously for IS*Pst9* (12).

The results obtained (Fig. 3A) suggested a cut-and-paste mechanism for mobilization for the IS*Ppu12* isoform as described for IS*Pst9* (12). In order to prove this, PCR amplifications using primers hybridizing with flanking regions of the IS*Ppu12* isoform in *P. stutzeri* AN10, FLANKPPU12-F and FLANKPPU12-R primers (Fig. 2A), were carried out to study the excision of the IS*Ppu12* isoform from its original localization. The expected PCR fragment of approximately 0.55 kb was obtained from AN10 transconjugants which had lost the IS*Ppu12* isoform from its original location (see Fig. S2 in the supplemental material), suggesting the excision of this IS. Six of these PCR DNA fragments were randomly selected and sequenced (see Fig. S2). The results showed that the IS*Ppu12* isoform excised imprecisely from its backbone DNA, taking from 4 to 11 flanking nucleotides in the process, as seen previously for IS*Pst9* (12). In one of the six transconjugants analyzed, the IS*Ppu12* isoform was not perfectly excised and one of the nucleotides of the IR remained in the DNA backbone of the transconjugant (see Fig. S2).

Next we analyzed if the IS*Ppu12* isoform of *P. stutzeri* AN10, like IS*Pst9* (12), also transposed by forming circular dsDNA transposition intermediates. A characteristic PCR product of 1.06 kb, the size expected if circular dsDNA transposition intermediates were forming, was obtained after contact with the conjugative *E. coli* S17-1λ_{pir} strain (but not with the nonconjugative DH5α strain) (see Fig. S3 in the supplemental material) using specific primers for the IS*Ppu12* isoform with outwards orientation (their location is shown in Fig. 2A): ISMG2, used also for IS*Pst9*, and ISPPU518R, specific for IS*Ppu12*. As previously shown for IS*Pst9* (12), the sequencing of the obtained PCR product revealed opposing IS*Ppu12* ends separated by 5 nucleotides, consisting in a combination of the IS flanking DNA in *P. stutzeri* AN10 (see Fig. S3).

The analysis of the sequence of IS*Pst9* did not reveal any region which could be putatively associated with regulation of transcription or with a well-defined function that could be easily tested (11). In contrast, two ORFs of the IS*Ppu12* isoform, *tnpR* (a *merR*-like transcriptional regulator) and *orf6* (a possible divalent heavy metal/H^+ antiporter), could be involved in heavy metal resistance. Therefore, we analyzed if the presence of the IS*Ppu12* isoform in the genome of *P. stutzeri* AN10 conferred any advantage for the bacterium when exposed to heavy metals and if the mobilization of IS*Ppu12* isoform was induced by these compounds. To do this, *P. stutzeri* AN10 and its derivative strain *P. stutzeri* AN10-5 harboring plasmid pDSK519 and lacking IS*L3*-like elements in its genome (Fig. 3A, lane 5) were grown with different concentrations of heavy metals and their responses were compared. No differences in MICs for different heavy metals were observed between the wild-type AN10 strain and the IS*L3*-like defective

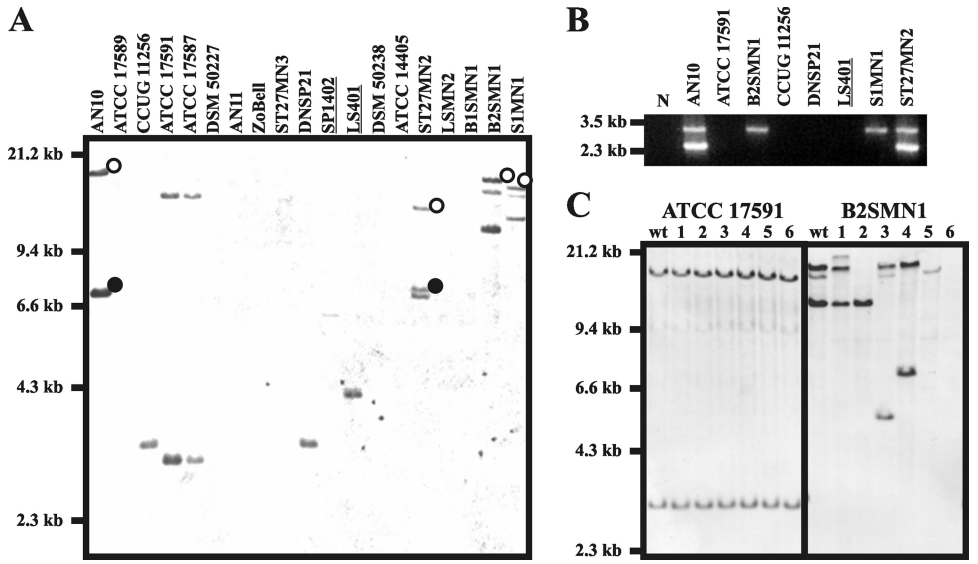

FIG. 4. IS*L3*-like element analysis in other *P. stutzeri* and *P. balearica* strains. (A) Southern blot hybridization with the *tnpA4* probe of EcoRI-digested genomic DNAs. White circles indicate those bands which also gave signals when hybridized with the *tnpR* probe (IS*Ppu12*), and black circles highlight those which gave signals when the *lysE* probe was used (IS*Pst9*). *P. balearica* strains are underlined. (B) PCR amplification products using primer IRISL3 and DNA from specified strains as template. (C) Southern blot hybridization with the *tnpA4* probe of EcoRI-digested genomic DNAs from wild type (wt) and six different derivatives that received plasmid pDSK519 by conjugation of the two specified *P. stutzeri* strains.

strain AN10-5 (MICs: Cd^{2+}, 16 mM; Co^{2+}, 128 mM; Cu^{+}, 512 mM; Hg^{2+}, 0.4 mM; Ni^{2+}, 256 mM; Pb^{2+}, 512 mM; and Zn^{2+}, 128 mM). To determine if the IS*Ppu12* isoform or IS*Pst9* increased its transposition frequency in the presence of the different metals, circular dsDNA transposition intermediate analysis of both ISs was done by PCR. Neither of the two ISs increased transposition activity in the presence of subinhibitory concentrations of the metals tested (results not shown).

Presence and mobilization of *P. stutzeri* AN10 IS*L3*-like elements in other *Pseudomonas* strains. Since the IS*Ppu12* isoform of *P. stutzeri* AN10 mobilized after conjugative interaction, the question arises as to whether or not this IS responded to the same stimulus in different bacteria. To answer this question, firstly we analyzed the mobilization of IS*Ppu12* in the strain in which this IS was originally described, *P. putida* mt-2 (39). As seen previously with the two IS*L3*-like ISs in *P. stutzeri* AN10, IS*Ppu12* circular dsDNA transposition intermediates were detected in *P. putida* mt-2 by PCR after conjugative interaction events with *E. coli* S17-1λ_{pir} (see Fig. S3 in the supplemental material). The nucleotide sequence of the obtained PCR product showed that, as in *P. stutzeri* AN10, the opposing IS*Ppu12* ends from *P. putida* mt-2 were separated by a combination of 5 bp flanking the IS (see Fig. S3). We also analyzed the mobilization of IS*Ppu12* in the genome of *P. putida* mt-2 transconjugants, which had received plasmid pDSK519 after conjugating with donor *E. coli* S17-1λ_{pir}. By using this plasmid as a genetic marker, we could ensure that there had been interaction between *P. putida* cells and the donor cells. Transconjugants were analyzed by Southern blot hybridization with *tnpA4* (the specific probe for both of the *P. stutzeri* AN10 IS*L3*-like elements). Surprisingly, only 3 out of 24 (12.5%) *P. putida* mt-2 transconjugants analyzed showed IS transposition (Fig. 3B), while over 93% of *P. stutzeri* AN10 transconjugants had shown IS*L3*-like transposition in the same type of experiment (Fig. 3A). As mentioned above, IS*Ppu12* of *P. putida* mt-2 differs from the *P. stutzeri* AN10 IS isoform in the sequence of a small region in the MerR-like transcriptional regulator TnpR. Thus, the reduced IS mobilization in *P. putida* mt-2 after conjugative interaction could be due to a less efficient activation by this mechanism caused by a degeneration of the MerR-like regulator. Alternatively, it could be caused by other cell factors involved in transposition, such IHF or FIS (23, 36), that could differ between the two strains or it could be due to the fact that the IS in *P. putida* mt-2 is located in a plasmid (39) and not in the chromosome as in *P. stutzeri* AN10.

Further mobilization analysis of the IS*L3*-like elements was done in other strains of *P. stutzeri* (16 strains) and *P. balearica* (2 strains), which is a closely related species. First of all we analyzed the presence of IS*L3*-like ISs in these strains. Southern blot hybridization with the *tnpA4* probe revealed the presence of 1 to 3 copies of IS*L3*-like ISs in 9 of the 18 strains analyzed (Fig. 4A). Hybridization with *lysE* (specific for IS*Pst9*) and *tnpR* (specific for IS*Ppu12*) probes showed that only two *P. stutzeri* strains, AN10 and ST27MN2, contained both IS*L3*-like ISs (Fig. 4A). Interestingly, strain ST27MN2 seemed to have an additional IS*L3*-like IS different from the characterized IS*Pst9* and IS*Ppu12* ISs. Similarly, 7 other strains revealed the plausible presence of noncharacterized IS*L3*-like ISs (Fig. 4A). Strains B2SMN1 and S1MN1 gave a signal with the *tnpR* probe, indicating that they might have IS*Ppu12*-like ISs. PCR analysis using degenerate primer IRISL3 was carried out with those strains that gave a positive *tnpA4* hybridization signal in order to confirm the presence of IS*Pst9* and IS*Ppu12* copies in their genomes. PCR amplifications confirmed the result obtained in the hybridization experiments (Fig. 4B). In *P. stutzeri* ST27MN2 a double band was obtained, as shown for strain

AN10. In *P. stutzeri* strains B2SMN1 and S1MN1 only the 3.4-kb band (IS*Ppu12*-like element) was observed. No amplification was obtained for the other 4 analyzed *tnpA4*-hybridizing strains (ATCC 17591, CCUG 11256, DNSP21, and LS401). The partial IS*Ppu12*-like sequences obtained from the three *P. stutzeri* strains (ST27MN2, B2SMN1, and S1MN1) were identical to the IS*Ppu12* isoform of AN10, especially when considering the variable region of *tnpR* (position 288 to 370) (see Fig. S4 in the supplemental material). The unique exception was *P. stutzeri* B2SMN1, which had 6 nucleotide differences in the 34-bp noncoding fragment between *tnpA* and the right IR. IS*Pst9*-like IS of strain ST27MN2 was also obtained and sequenced (see Fig. S4). Only 2 nucleotide differences were found in the 876-bp sequence compared with IS*Pst9* of *P. stutzeri* AN10, but both nucleotide changes led to no apparent amino acid variation (results not shown).

We then analyzed if the IS*L3*-like ISs present in these strains mobilized after conjugative interaction. Mating experiments with *E. coli* S17-1λ_{pir} harboring pDSK519 and further Southern blot hybridization analysis of transconjugants revealed (as shown for B2SMN1 in Fig. 4C) that strains harboring the *tnpR*-containing IS*Ppu12*-like IS mobilized all their *tnpA4*-containing IS*L3*-like ISs after conjugative interaction. On the other hand, no transposition was detected in transconjugants derived from strains without a *tnpR*-containing IS*Ppu12*-like IS (such as ATCC 17591 in Fig. 4C). The formation of circular dsDNA transposition intermediates was analyzed in those strains (B2SMN1, S1MN1, and ST27MN2) shown to have IS*Pst9*-like and/or IS*Ppu12*-like ISs. PCR product revealing the formation of IS*Ppu12* circular dsDNA transposition intermediates was obtained in all three strains (results not shown). As expected, the sequence of the amplicons showed the presence of opposing IS*Ppu12* ends separated by 5 random base pairs (results not shown) as previously observed in *P. stutzeri* AN10 and in *P. putida* mt-2. This means that there was a common mechanism for excision of these ISs in the *P. stutzeri* strains tested as well as in *P. putida* mt-2.

After all these experiments it seemed clear that mobilization of IS*L3*-like ISs after conjugative interaction with *E. coli* S17-1λ_{pir} was not exclusive to *P. stutzeri* AN10 but was a more general phenomenon that occurred in other *P. stutzeri* strains as well as in other *Pseudomonas* species. Horizontal DNA transfer by conjugation is found in many prokaryotes (17), but cell-to-cell contact is not always "friendly," since hostile conjugation processes have been described (40). In fact, a conjugative process for exchange of genetic material between cells could be considered a stressful situation for the receptor cell, even more so when conjugal transfer systems have been evolutionarily related to pathogenic export of DNA and proteins across bacterial membranes (40). If conjugation leads to a stress response in the receptor cell, the mobilization of ISs is not unexpected, as it happens under other stress conditions such as starvation (22). Several cell-to-cell contact-dependent signaling mechanisms have been reported (reviewed in reference 4). Conjugative interaction might be a contact event triggering a very specific signaling cascade in the receiving cell yet to be determined. But since conjugation is common to many different bacteria, it would be reasonable to think that there might be a common cellular response to a mating event which results in the activation signal for transposition of these ISs. A biological significance for the IS mobilization might be increasing the likelihood of its dispersion among bacteria or a mechanism that the host cell uses for stress defense.

IS*Pst9* and IS*Ppu12* independent transposition. Our results showed that IS*Pst9* and IS*Ppu12* mobilized after conjugative interaction when both were present in the genome of *P. stutzeri* strains (i.e., in AN10 and ST27MN2 [Fig. 3A and 4B]). On the other hand, the results obtained with *P. putida* mt-2 and with *P. stutzeri* strain B2SM1 showed that an IS*Ppu12*-like IS mobilized after conjugative interaction in the absence of an IS*Pst9*-like IS (Fig. 3B and 4C). But we did not know if IS*Pst9* would mobilize after conjugative interaction in the absence of IS*Ppu12*. In order to determine if IS*Pst9* and IS*Ppu12* could transpose independently after conjugative interaction, both ISs were cloned in pBBR1MCS-5, a replicative plasmid in *Pseudomonas*, resulting in pJOC40 (with IS*Pst9*) and pJOC41 (with IS*Ppu12* isoform of *P. stutzeri* AN10). Both plasmids were introduced independently by conjugation in two different genetic backgrounds: *P. stutzeri* AN11 (a strain which does not have any IS*L3*-like ISs as shown in Fig. 4A) and AN10-5 (a *P. stutzeri* AN10 transconjugant harboring plasmid pDSK519, which had lost all IS*L3*-like copies as shown in Fig. 3A, lane 5). Three different isolates of each strain harboring pJOC40 or pJOC41 plasmids were used for further conjugative interaction events with *E. coli* S17-1λ_{pir} to activate IS transposition from the plasmids. Surprisingly, formation of circular dsDNA transposition intermediates was observed only in both strains containing pJOC41 with IS*Ppu12*, whereas no amplification product was detected for those containing pJOC40 with IS*Pst9* (Fig. 5A).

Further analysis for independent transposition activation was done using four *P. stutzeri* AN10 transconjugants harboring pDSK519 (Fig. 3A) which presented both IS*L3*-like ISs (AN10-3, -8, -19, and -21), four which conserved only IS*Pst9* (AN10-4, -9, -11, and -28), and four with only the IS*Ppu12* isoform (AN10-6, -15, -17, and -20). Contact events with *E. coli* S17-1λ_{pir} were performed for each one of the 12 transconjugants, and formation of circular dsDNA transposition intermediates was examined by multiplex PCR (Fig. 5B). Transconjugants with both IS*L3*-like elements gave, as for the wild type, PCR amplification for circular dsDNA transposition intermediates of both ISs, IS*Pst9* and IS*Ppu12* isoform. Transconjugants with only IS*Ppu12* isoform copies gave PCR amplification of circular dsDNA transposition intermediates for this element and, as expected, not for IS*Pst9*. Formation of circular dsDNA transposition intermediates was not detected in those transconjugants containing only IS*Pst9*. The kinetics of formation of circular dsDNA transposition intermediates for both IS*L3*-like elements was followed in *P. stutzeri* AN10 after contact with *E. coli* S17-1λ_{pir}. A 25-cycle multiplex PCR was done using total DNA obtained after different incubation times as template. Semiquantification from three independent experiments revealed a delay in formation of IS*Pst9* circular dsDNA transposition intermediates of approximately 15 min in relation to that of the IS*Ppu12* isoform (Fig. 5C). All these results show that IS*Pst9* does not have the ability to upregulate its transposition after conjugative interaction by itself and that the IS*Ppu12* isoform is the IS element activated by conjugative interaction. Upon activation, IS*Ppu12* can cause transposition of other IS elements with similar IRs, like IS*Pst9*. However,

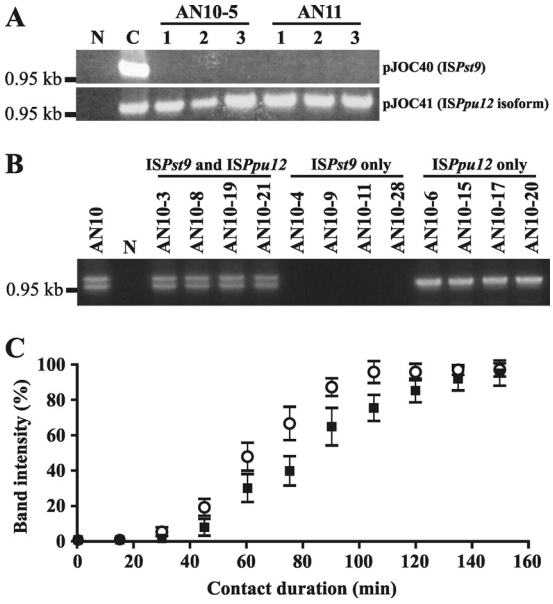

FIG. 5. IS*Ppu12* isoform and IS*Pst9* independent transposition and kinetics. (A) PCR amplification of circular dsDNA transposition intermediates from plasmids pJOC40 (primers ISMG2 and ISMG4) and pJOC41 (primers ISMG2 and ISPPU518R) harbored in three different AN10-5(pDSK519) and AN11 isolates after induction by conjugative interaction with *E. coli* S17-1λ_{pir}. A negative PCR control (N) and a positive control (C) using the AN10 strain after interaction with *E. coli* S17-1λ_{pir} are shown. (B) PCR amplification of circular dsDNA transposition intermediates formed in different AN10 transconjugants harboring pDSK519 (Fig. 3A) and induced by *E. coli* S17-1λ_{pir} interaction. Primers ISMG2, ISMG4, and ISPPU518R were used. The presence of IS*Pst9* and IS*Ppu12* in the different AN10 transconjugants is indicated. A positive control using the AN10 strain and a PCR negative control (N) are shown. (C) Semiquantification of IS*Pst9* (black squares) and IS*Ppu12* (white circles) formation of circular dsDNA transposition intermediates from three independent contact experiments between *P. stutzeri* AN10 and *E. coli* S17-1λ_{pir} at different incubation times.

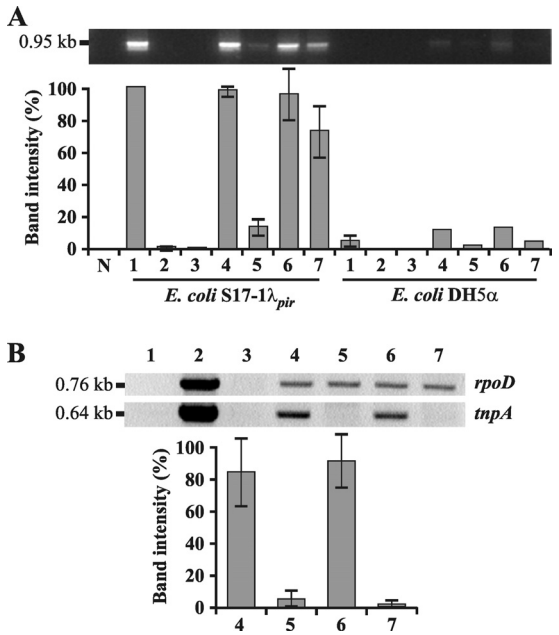

FIG. 6. (A) TnpR regulation of formation of circular dsDNA transposition intermediates. Experiments represented are as follows: 1, AN10 wild-type strain; 2, AN11 wild-type strain; 3, AN11(pJOC42); 4, AN11(pJOC41); 5, AN11(pJOC41mut); 6, AN11(pJOC41, pJOC42); 7, AN11(pJOC41mut, pJOC42). pJOC41 harbored the intact IS*Ppu12* isoform of *P. stutzeri* AN10, pJOC41mut harbored a *tnpR* mutant of the IS, and pJOC42 harbored the *tnpR* complementation. Four-hour mating events were carried out with the *E. coli* strains S17-1λ_{pir} (pilus-forming strain) and DH5α (non-pilus-forming strain). A PCR negative control is also represented (N). For analysis, the highest measured signal was defined as 100%. Standard deviations of three independent experiments are shown. (B) *tnpA* and *rpoD* PCR amplification using cDNAs obtained from different mating events between *P. stutzeri* and *E. coli*. Lanes: 1, PCR negative control; 2, PCR positive control using total genomic DNA from *P. stutzeri* AN10; 3, *E. coli* PCR negative control for *tnpA* and *rpoD* amplification; 4 and 5, AN10 mated with *E. coli* strains S17-1λ_{pir} and DH5α, respectively; 6 and 7, AN11 containing plasmids pJOC41 and pJOC41mut, respectively, mated with *E. coli* strain S17-1λ_{pir}. The graph represents semiquantification of *tnpA* amplification referred to the signal obtained for the *rpoD* gene. Standard deviations from three independent mating experiments are shown.

mobilization of IS*Pst9* in the absence of IS*Ppu12* has been observed previously (11), and therefore, IS*Pst9* is able to respond to so-far-undetermined stimuli different from conjugative interaction in *P. stutzeri*.

TnpR of the IS*Ppu12* isoform upregulates transposition after conjugative interaction events. Since TnpR showed high identities with MerR-like transcriptional regulator proteins and there was a plausible conserved MerR-like DNA binding region (5′-TTGACC-N_{19}-TTAAAT-3′) (9) located between *tnpR* and *orf6*, with an *orf6-lspA-tnpA* orientation (coordinates 527 to 557 of sequence FJ624110 [Fig. 2A and B]), we hypothesized that TnpR might have a role in regulating the transposition of IS*Ppu12* after conjugative interaction. To answer this question, we generated a broad-host-range plasmid containing a *tnpR*-defective IS*Ppu12* IS by deletion of 8 bp located 39 nucleotides downstream of the ATG starting codon of *tnpR* (plasmid pJOC41mut). The *tnpR* mutation resulted in an in-frame disruption after only 51 bp, allowing the transcription and further translation of a 17-amino-acid (aa)-long peptide with no expected activity. A *P. stutzeri* replicative plasmid (compatible with pJOC41mut) harboring the native *tnpR* gene was constructed (pJOC42) to complement the *tnpR* mutant genotype.

Given the fact that the IS*Ppu12* isoform in pJOC41 transposed in *P. stutzeri* AN11 after conjugative cell interaction (Fig. 5A), this strain was used for *tnpR* functional analysis. As early data showed that different *merR*-like genes could complement each other (16), *P. stutzeri* AN11 was analyzed previously in order to rule out the presence of other possible *tnpR*-like genes in its genome. This strain gave no hybridization signal when *tnpR* was used as a probe in Southern blot hybridization (results not shown).

Plasmids pJOC41 (IS*Ppu12*, *tnpR*$^+$) and pJOC41mut (IS*Ppu12*, *tnpR* negative) were independently introduced by conjugation into *P. stutzeri* AN11. The resulting strains were complemented with the native *tnpR* gene by introducing plasmid pJOC42 in them. Assays of separate contact events between the generated strains and their ancestor with *E. coli* strains with (strain S17-1λ_{pir}) or without (strain DH5α) the ability to produce conjugative pili were performed (Fig. 6A). As expected, formation of basal circular dsDNA transposition intermediates was observed when AN10 (used here as a con-

trol) was mated with DH5α, and no signal was obtained in any case when *P. stutzeri* strain AN11 or its derivative harboring only the pJOC42 plasmid (*tnpR* gene) was used. *P. stutzeri* AN11 derivatives harboring plasmid pJOC41 (IS*Ppu12*, *tnpR*$^+$) behaved like strain AN10, independently of the presence of plasmid pJOC42. In contrast, *P. stutzeri* AN11 harboring pJOC41mut (IS*Ppu12*, *tnpR* negative) presented basal levels of formation of circular dsDNA transposition intermediates and there was a 72% recovery of formation of circular dsDNA transposition intermediates when the strain was complemented with the pJOC42 plasmid (Fig. 6A). These results seemed to indicate that *tnpR* was directly involved in activating IS transposition after conjugative interaction.

One possible hypothesis was that *tnpR* regulated the expression of *tnpA*, the transposase gene of the IS*Ppu12* isoform, and therefore, this possibility was explored. First, the transcription of the *tnpA* gene in strain *P. stutzeri* AN10 was analyzed by obtaining cDNA from different mating events with *E. coli* S17-1λ_{pir} and DH5α strains. *tnpA* cDNA amplification was observed only in the presence of strain S17-1λ_{pir} (Fig. 6B, lanes 4 and 5) in agreement with the results showing the mobilization of this IS after conjugative interaction with this strain but not with DH5α. As a control for this experiment, we analyzed the expression of a housekeeping gene, *rpoD*, which should not be affected by the interaction, and no differences in its expression were obtained for mating events of *P. stutzeri* with both *E. coli* strains. Then, *tnpA* transcription was analyzed in *P. stutzeri* AN11 carrying pJOC41 (IS*Ppu12*, *tnpR*$^+$) or pJOC41mut (IS*Ppu12*, *tnpR* negative) after mating with *E. coli* S17-1λ_{pir}. cDNA amplification was obtained only in AN11 carrying pJOC41, but not in the *tnpR* mutant (Fig. 6B, lanes 6 and 7). These results demonstrated that TnpR was involved in the upregulation of the transcription of IS*Ppu12* transposase after conjugative interaction.

Sequence analysis of TnpR revealed that its N-terminal DNA binding domain conserved a high homology with other MerR-like regulators. On the other hand, the C-terminal region, known as the effector binding domain (9), shared very little similarity to other regulators of this family, although the three conserved cysteines (Cys77, Cys112, and Cys121) in charge of stabilizing the binding of the activating heavy metal were present. Thus, TnpR of IS*Ppu12* may function similarly to the transcriptional regulators of the MerR family, except that in this case the effector could be an intermediate element from the specific conjugative interaction signaling cascade. The MerR family of transcriptional regulators is known to bind permanently to the operator site weakly repressing transcription in the absence of the stimulus (9). The absence of TnpR would result in basal *tnpA* transcription, which explains the low circular dsDNA transposition intermediate formation activity observed in strain AN11 harboring plasmid pJOC41mut (Fig. 6A, lane 5). The fact that basal *tnpA* transcription can happen with no regulator could explain the transposition events even after *tnpR* mutation, as observed by Williams and coworkers (39). MerR-like regulators are strongly activated with low concentrations of inductor and cause highly upregulating transcription (14, 31). We propose that TnpR, probably linked to the MerR-like DNA binding region found in IS*Ppu12*, could activate *tnpA* transcription during conjugation conditions, increasing the production of TnpA enzyme and, consequently, of transposition (see Fig. S5 in the supplemental material).

Concerning the mechanism for the activation in *trans* of IS*Pst9* transposition by IS*Ppu12*, two possibilities can be proposed: a transcriptional regulation effect of TnpR over the IS*Pst9* transposase or a direct transposition activity of the IS*Ppu12* transposase after recognition of the IRs of IS*Pst9*, which are nearly identical (Fig. 2D). To discard the first of these possibilities, *P. stutzeri* AN11 harboring pJOC40 (plasmid containing IS*Pst9*) was complemented with pJOC42 (plasmid with *tnpR*). No PCR amplification of IS*Pst9* circular dsDNA transposition intermediates was obtained after contact events between AN11(pJOC40, pJOC42) and *E. coli* S17-1λ_{pir}. This result ruled out the possibility of a direct TnpR regulating effect on IS*Pst9* transposition, and therefore, the most likely explanation seems to be the mobilization of IS*Pst9* directly mediated by the TnpA of the IS*Ppu12* isoform (see Fig. S5 in the supplemental material).

Although further experiments would be required to characterize the contact-dependent signaling after conjugative interaction and to fully characterize the TnpR effect after mating events, we can conclude that this transcriptional regulator seems to be an intermediate step in the signal cascade originating after cell mating that enhances IS*Ppu12* transposition and, indirectly, IS*Pst9* mobilization.

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