TnpR Encoded by an ISPpu12 Isoform Regulates Transposition of Two Different ISL3-Like Insertion Sequences in Pseudomonas stutzeri after Conjugative Interaction

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Received 9 October 2009/Accepted 29 December 2009

Pseudomonas stutzeri AN10 has two ISL3-like insertion sequences (ISs). One of them has been recently described as ISPst9. In this study we show that the second IS, situated 4.5 kb upstream of ISPst9, is an isoform of ISPpu12 from Pseudomonas putida mt-2. Although both ISL3-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 ISPst9, the isoform of ISPpu12 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 S17-1λarp (conjugative interaction) but not with the nonconjugative E. coli DH5α. In fact, we demonstrate that ISPst9 transposition after conjugative interaction occurs only when ISPpu12 is present, indicating that ISPpu12 is upregulating transposition of both ISs under such conditions. We also demonstrate that this conjugative interaction-mediated induction of ISPpu12 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 ISPpu12 but not in ISPst9, reduced the transcription of tnpA (ISPpu12 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 ISPpu12-free genetic background did not induce ISPst9 after conjugative interaction. Thus, our results suggest that TnpR, after conjugative interaction, activates transcription of tnpA of ISPpu12. Then, TnpA of ISPpu12 would bind to IRs of both ISs, ISPpu12 and ISPst9, 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): nahABFCEGD (nah upper pathway), coding for the enzymes involved in the conversion of naphthalene to salicylate; nahGTHINLOMKI (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 ISL3 family, ISPst9, was identified and characterized (11). In P. stutzeri, ISPst9 was involved in catabolic gene inactivation (interrupting gene nahH), like two of its closest relatives in the ISL3 family, ISPst2 from Pseudomonas sp. OX1 (5) and ISPpu12 from Pseudomonas putida mt-2 (39). ISPst9 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 ISPst9 transposase gene (tnpA4) revealed the presence of a putative second ISPst9-like copy in P. stutzeri AN10 (12). Both ISL3-like elements of P. stutzeri AN10 had the peculiarity of increasing their transposition frequency after conjugative interaction with an Escherichia coli pilus-producing strain (S17-1λarp), 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 ISL3-like elements present in P. stutzeri AN10 were proximate in its

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† Supplemental material for this article may be found at http://jb.asm.org/.
‡ Published ahead of print on 8 January 2010.
nying (one of the three ORFs accompanying the 14405, DNSP21, DSM 50227, DSM 50238, LSMN2, ST27MN3, S1MN1, and coli L3). In this work we show that the second IS-Like copies were in fact different. isoform is recommended by each manufacturer. Except when specified, PCR amplifications an 0.5-kb product in both cases. Plasmid DNA was isolated by alkaline lysis using the spotted together onto a membrane filter (nitrocellulose, 0.22 mm; Millipore) performing conjugative interaction events with different incubation times as P. stutzeri AN10 corresponds to an IS-Like ISs in P. stutzeri AN10 before 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 ISPPu12 isofrom.

**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-SiPPuDSK519 (12); ATCC 17598, ATCC 17580, ATCC 17591, and DSM 51226 (35). ATCC 14405, DSNP21, DSM 50227, DSM 50238, DSM 50275, and DSM 50271 (32). BSM1N1, BSM2N1, and ST27N2 (33). P. putida mt-2 (38) and Pseudomonas bariolae LS401 and SPI1402 (3) were also used. The conjugative E. coli S17-1&agr;&agr;&agr; (21), which has the tr 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 minimal basal medium (MMB) (2) supplemented with 0.5% (wt/vol) sucrose. 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 all metals were calculated as previously described (13) and used for inoculation of LB (M. Mayer) supplemented with 0.5% (wt/vol) succinate and the respective heavy metal salt [CoCl₂·(CH₃COO)₂Pb·3H₂O, CoCl₂·6H₂O, CuCl₂·2H₂O, HgCl₂, NiCl₂·6H₂O, or ZnCl₂].

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, IS94, for the transposase-encoding gene in IS-Like elements was obtained using ISMG3 and ISMG9 primers (11). Specific probes for detection of IS909 and ISPPu12 were made by PCR as follows: 94°C for 5 min; 30 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, and 72°C for 10 min. The 5.0-kb amplicon obtained was cloned in pCR2.1 (TOPO-TA cloning kit; Invitrogen), giving pJOC42. Both IS-Like elements of Pseudomonas strains, IS909 and ISPPu12, were performed using primers IRISL3 (5'-GGGTAAGACGCTAT-3') and IRISL4 (5'-AGAATCCGTTGGGGAGGAGG-3'), located at the intermediate region between IS and the intermediate region 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 program (SynGene). 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 DNA-treated reaction 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 recommendations. Semiquantification of mRNA was performed using primers ISMG3 and ISMG9 and conditions described previously (12). Amplification of the housekeeping gene ptod, using primers pEG30F and pEG790R and conditions described elsewhere (28), was done for signal normalization.

**Plasmid construction.** PCR amplification of the IS-Like element intermediate region was carried out using primers ISMG2 and ISMG4 (21) 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-Like elements of Pseudomonas strains, IS909 and ISPPu12, were performed using primers IRISL3 (5'-GGGTAAGACGCTAT-3') and IRISL4 (5'-AGAATCCGTTGGGGAGGAGG-3'), located at the intermediate region between IS and IS. 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 pTOPOPp9 and pTOPOPp12. 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 IS909) and pJOC41 (with ISPPu12). The mpf-defective ISPPu12 IS was obtained by amplifying plasmid pTOPOPp12 PCR using primers IRISL3 (5'-CGGCGCACTGAGCCGTTG-3') and IRISL4 (5'-CGGCGCACTGAGCCGTTG-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; and 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 pTOPOPp12mut by an EcoRI digestion and cloned in the EcoRI-linearized pBBR1MCS-5, producing pJOC41mut. Complementation of mpfR was achieved by cloning the PCR amplification of a 1.7-kb fragment of ISPPu12 of strain AN10 (containing mpfR in pCR2.1 (TOPO-TA cloning kit; Invitrogen), producing pTOPOPp12mut, pTOPOPp12mut. Isolating was done by cloning pTOPOPp12mut by an 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 ISPPu12 of P. stutzeri AN10 and the intermediate region between ISPPu12 and ISPPu9 was determined directly from plasmids pTOPOPp12 and pJOC26 by using the primer walking method, with the design of new primers based on the determined sequences. To obtain the DNA sequence of ISPPu12, an inverse PCR was carried out. For this, genomic DNA from P. stutzeri AN10 was digested with Smal. The digestion was purified (PureLink PCR purification kit; Invitrogen) and autoligated. The ligation product was used as template for PCR amplification using ENTF3E (5'-CTATGCGGCAAAGTCCCATCGC-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 ISPst9-like copy of P. stutzeri AN10.** Weightman et al. (37) described how two copies of ISPpu12, a close relative of ISPst9, formed a functional composite catabolic transposon, named DEH in *P. putida* PP3. Given the facts that (i) *P. stutzeri* AN10 presented two plausible ISPst9 copies revealed by Southern blot hybridization (12), (ii) ISPst9 occurred in bacteria together with the presence of aromatic hydrocarbon-degrading determinants (11), and (iii) the ISPst9 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 ISPst9-like copies in *P. stutzeri* AN10 constituted a composite catabolic transposon similar to DEH.

In order to evaluate if both ISPst9-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 ISPst9, *tnpA4*, revealed that both ISPst9-like copies coexisted in a single 15-kb StuI-StuI DNA fragment and also in a single 20-kb Xhol-XhoI DNA fragment (Fig. 1A). According to the known location of the StuI and XhoI restriction sites in the ISPst9 flanking sequence (7, 11), the hybridization results suggested that both ISPst9-like ISs were separated by an intermediate DNA sequence of 4 to 6 kb and that the second ISPst9-like element was situated upstream of the originally described copy of ISPst9 (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 ISPst9 (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 pTOPOPpu12 confirmed that the second ISPst9 copy previously localized in the chromosome of *P. stutzeri* AN10 (3.4- and 2.5-kb bands) and *P. putida* mt-2 (3.4-kb band) DNAs were used as templates.

![FIG. 1.](http://jb.asm.org/)

**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 ISPst9), whereas white circles indicate those which gave signals when hybridized with the *tnpR* probe (for ISPpu12). (B) Agarose gel showing the 5.0-kb amplified product corresponding to the intermediate region between the two ISL3-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 ISPst9 of *P. stutzeri* AN10 (11). This result suggested that the second ISPst9-like IS present in *P. stutzeri* AN10 could be an ISPpu12 isoform.

In order to complete the DNA sequence of this plausible ISPpu12 isoform, a degenerate primer (IRISL3) was designed. The IRISL3 primer hybridized specifically with the inverted repeat (IR) sequences of ISPst9 and ISPpu12 and was used to amplify by PCR both ISL3-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 ISPst9 (2.5 kb) (11) and another with the expected size for ISPpu12 (3.4 kb) (39). Both PCR products were cloned in pCR2.1-TOPO (Invitrogen), resulting in pTOPOPpu12 and pTOPOPpu12 plasmids, respectively, and both DNA inserts were sequenced. The 2,472-bp nucleotide sequence corresponding to the pTOPOPpu12 insert was 100% identical to the previously sequenced ISPst9 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 ISPst9-like IS of *P. stutzeri* AN10 was an isoform of ISPpu12 (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 ISPpu12 isoform of *P. stutzeri* AN10 contained four ORFs (Fig. 2B): *tnpA4*, which codes for the transposase; *lysA*, 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 Pout...
resided in a small region of the pnpR
isoform and IS differences were observed between the
P. stutzeri (Fig. 2B) (39) was also perfectly conserved. Only 18 nucleotide
transcriptional regulator.

at the end of the DNA binding domain of the plausible TnpR
nucleotide differences caused 6 amino acid variations (Fig. 2C)
and consequent amino acid differences (marked by boldface and asterisks) between the IS
isoform transposes in
P. stutzeri

(TDRs) were flanking the IS
isoform in AN10, although

(Fig. 2B) (39) was also perfectly conserved. Only 18 nucleotide
differences were observed between the P. stutzeri AN10 IS
Ppu12 isoform and ISppu12 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 ISpst9 and ISppu12 isoforms of P. stutzeri AN10 were their transposase-encoding genes and their IR sequences. Transposases of the two ISs shared 90.4% nucleotide identity, which explained why two ISs hybridized against the genes showed 90.4% nucleotide identity, which

an AT-rich sequence was observed at both sides.

P. putida
mt-2

P. stutzeri
AN10

FIG. 2. (A) Schematic representation of the two ISL3-like ISs in P. stutzeri AN10 (ISppu12 and ISpst9). 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 ISppu12 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 ISppu12 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 ISppu12 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 ISsst9 and ISppu12.

(Fig. 2B) 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 ISppu12 isoform in AN10, although an AT-rich sequence was observed at both sides.

ISppu12 isoenzymes transposes in P. stutzeri AN10 similarly to ISsst9. The mobilization of ISsst9 in P. stutzeri AN10 was found to be induced after conjugative interaction with the pili-producing strain E. coli S17-1lpw (12). Therefore, in order to analyze the mechanisms of mobilization of the ISppu12 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 S17-1lpw revealed that 93.3% of them (28 out 30) changed the original tnpA4 hybridization pattern. Since our new results show that both ISs (ISsst9 and ISppu12 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 ISppu12 isoform (tnpR probe) and for ISsst9 (lysE probe) to differentiate which bands corresponded to each one of the two ISL3-like elements. The two IS elements, ISsst9 and ISppu12, 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.
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).

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 S17-1p pir strain (but not with the non-conjugative 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 ISL3-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 ISL3-like defective...
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 ISPpu12 isoform or ISPst9 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 ISL3-like elements in other *Pseudomonas* strains.** Since the ISPpu12 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 ISPpu12 in the strain in which this IS was originally described, *P. putida* mt-2 (39). As seen previously with the two ISL3-like ISs in *P. stutzeri* AN10, ISPpu12 circular dsDNA transposition intermediates were detected in *P. putida* mt-2 by PCR after conjugative interaction events with *E. coli* S17-1Apar (see Fig. S3 in the supplemental material). The nucleotide sequence of the obtained PCR product showed that, as in *P. stutzeri* AN10, the opposing ISPpu12 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 ISPpu12 in the genome of *P. putida* mt-2 transconjugants, which had received plasmid pDSK519 after conjugating with donor *E. coli* S17-1Apar. 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 ISL3-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 ISL3-like transposition in the same type of experiment (Fig. 3A). As mentioned above, ISPpu12 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 as 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 ISL3-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 ISL3-like ISs in these strains. Southern blot hybridization with the *tnpA4* probe revealed the presence of 1 to 3 copies of ISL3-like ISs in 9 of the 18 strains analyzed (Fig. 4A). Hybridization with *lysE* probe (specific for ISPst9) and *tnpR* (specific for ISPpu12) probes showed that only two *P. stutzeri* strains, AN10 and ST27MN2, contained both ISL3-like ISs (Fig. 4A). Interestingly, strain ST27MN2 seemed to have an additional ISL3-like IS different from the characterized ISPst9 and ISPpu12 ISs. Similarly, 7 other strains revealed the plausible presence of noncharacterized ISL3-like ISs (Fig. 4A).

Strains B2SMN1 and SIMN1 gave a signal with the *tnpR* probe, indicating that they might have ISPpu12-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 ISPst9 and ISPpu12 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 (ISpusu12-like element) was observed. No amplification was obtained for the other 4 analyzed tnpA4-hybridizing strains (ATCC 17591, CCUG 11256, DSNP21, and LS401). The partial ISpusu12-like sequences obtained from the three P. stutzeri strains (ST27MN2, B2SMN1, and S1MN1) were identical to the ISpusu12 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 tnpR and the right IR. ISPst9-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 ISpusu9 of P. stutzeri AN10, but both nucleotide changes led to no apparent amino acid variation (results not shown).

We then analyzed if the ISL3-like ISs present in these strains mobilized after conjugative interaction. Mating experiments with E. coli S17-1λpir, harboring pDSK519 and further Southern blot hybridization analysis of transconjugants revealed (as shown for B2SMN1 in Fig. 4C) that strains harboring the tnpR-containing ISpusu12-like IS mobilized all their tnpA4-containing ISL3-like ISs after conjugative interaction. On the other hand, no transposition was detected in transconjugants derived from strains without a tnpR-containing ISpusu12-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 ISPst9-like and/or ISpusu12-like ISs. PCR product revealing the formation of ISpusu12 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 ISpusu12 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 ISL3-like ISs after conjugative interaction with E. coli S17-1λpir 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 conjugation 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.

**ISPs9 and ISPp12 independent transposition.** Our results showed that ISPs9 and ISPp12 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 ISpusu12-like IS mobilized after conjugative interaction in the absence of an ISPs9-like IS (Fig. 3B and 4C). But we did not know if ISPs9 would mobilize after conjugative interaction in the absence of ISPp12. In order to determine if ISPs9 and ISPp12 could transpose independently after conjugative interaction, both ISs were cloned in pBRR1MC5-5, a replicative plasmid in Pseudomonas, resulting in pJOC40 (with ISPs9) and pJOC41 (with ISPp12 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 ISL3-like ISs as shown in Fig. 4A) and AN10-5 (a P. stutzeri AN10 transconjugant harboring plasmid pDSK519, which had lost all ISL3-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 S17-1λpir to activate IS transcription from the plasmids. Surprisingly, formation of circular dsDNA transcription intermediates was observed only in both strains containing pJOC41 with ISPp12, whereas no amplification product was detected for those containing pJOC40 with ISPs9 (Fig. 5A).

Further analysis for independent transposition activation was done using four P. stutzeri AN10 transconjugants harboring pDSK519 (Fig. 3A) which presented both ISL3-like ISs (AN10-3, -8, -19, and -21), four which conserved only ISPs9 (AN10-4, -9, -11, and -28), and four with only the ISPp12 isoform (AN10-6, -15, -17, and -20). Contact events with E. coli S17-1λpir were performed for each one of the 12 transconjugants, and formation of circular dsDNA transcription intermediates was examined by multiplex PCR (Fig. 5B). Transconjugants with both ISL3-like elements gave, as for the wild type, PCR amplification for circular dsDNA transcription intermediates of both ISs, ISPs9 and ISPp12 isoform. Transconjugants with only ISPp12 isoform copies gave PCR amplification of circular dsDNA transcription intermediates for this element and, as expected, not for ISPs9. Formation of circular dsDNA transposition intermediates was not detected in those transconjugants containing only ISPs9. The kinetics of formation of circular dsDNA transposition intermediates for both ISL3-like elements was followed in P. stutzeri AN10 after contact with E. coli S17-1λpir. 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 ISPs9 circular dsDNA transposition intermediates of approximately 15 min in relation to that of the ISPp12 isoform (Fig. 5C). All these results show that ISPs9 does not have the ability to upregulate its transposition after conjugative interaction by itself and that the ISPp12 isoform is the IS element activated by conjugative interaction. Upon activation, ISPp12 can cause transposition of other IS elements with similar IRs, like ISPs9. However,
mobilization of ISp9 in the absence of ISp12 has been observed previously (11), and therefore, ISp9 is able to respond to so-far-undetermined stimuli different from conjugative interaction in P. stutzeri.

**TnpR of the ISp12 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-N19-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 ISp12 after conjugative interaction. To answer this question, we generated a broad-host-range plasmid containing a tnpR-defective ISp12 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 (plasmid pJOC41mut). 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.

### FIG. 5

**ISp12 isoform and ISp9 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 S17-1λpir. A negative PCR control (N) and a positive control (C) using the AN10 strain after interaction with E. coli S17-1λpir are shown. (B) PCR amplification of circular dsDNA transposition intermediates formed in different AN10 transconjugants harboring pDSK519 (Fig. 3A) and induced by E. coli S17-1λpir interaction. Primers ISMG2, ISMG4, and ISPPU518R were used. The presence of ISp9 and ISp12 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 ISp9 (black squares) and ISp12 (white circles) formation of circular dsDNA transposition intermediates from three independent contact experiments between P. stutzeri AN10 and E. coli S17-1λpir 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, pJOC42); 5, AN11 (pJOC41mut, pJOC42); 7, AN11 (pJOC41mut, pJOC42). pJOC41 harbored the intact ISp12 isoform of P. stutzeri AN10, pJOC41mut harbored a tnpR mutant of the IS, and pJOC42 harbored the npr complementation. Four-hour mating events were carried out with the E. coli strains S17-1λpir (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 S17-1λpir and DH5α, respectively; 6 and 7, AN11 containing plasmids pJOC41 and pJOC41mut, respectively, mated with E. coli strain S17-1λpir. 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.
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 (ISPpu12, tnpR+) behaved like strain AN10, independently of the presence of plasmid pJOC42. In contrast, P. stutzeri AN11 harboring pJOC41mut (ISPpu12, 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 ISPpu12 isofrom, 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 S17-1λpir and DH5α strains. tnpA cDNA amplification was observed only in the presence of strain S17-1λpir (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 (ISPpu12, tnpR+) or pJOC41mut (ISPpu12, tnpR- negative) after mating with E. coli S17-1λpir. 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 ISPpu12 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 ISPpu12 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 transcription events even after tnpR mutation, as observed by Williams and coworkers (39). MerR-like regulators are strongly activated with low concentrations of inducer and cause highly upregulating transcription (14, 31). We propose that TnpR, probably linked to the MerR-like DNA binding region found in ISPpu12, 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 ISPst9 transposition by ISPpu12, two possibilities can be proposed: a transcriptional regulation effect of TnpR over the ISPst9 transposase or a direct transposition activity of the ISPpu12 transposase after recognition of the IRSs of ISPst9, which are nearly identical (Fig. 2D). To discard the first of these possibilities, P. stutzeri AN11 harboring pJOC40 (plasmid containing ISPst9) was complemented with pJOC42 (plasmid with tnpR). No PCR amplification of ISPst9 circular dsDNA transposition intermediates was obtained after contact events between AN11(pJOC40, pJOC42) and E. coli S17-1λpir. This result ruled out the possibility of a direct TnpR regulating effect on ISPst9 transposition, and therefore, the most likely explanation seems to be the mobilization of ISPst9 directly mediated by the TnpA of the ISPpu12 isofrom (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 ISPpu12 transposition and, indirectly, ISPst9 mobilization.

ACKNOWLEDGMENTS

J.A.C.-O. was supported by a grant from the C.A.I.B. (Government of the Balearic Islands). Funds were obtained from projects CTM-2005-01783 and CTM2008-02574 from M.E.C. (Spanish Ministry of Education and Science), both with FEDER cofunding. We thank Kenneth N. Timmis for critically reading the manuscript.

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