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ISPst9, an ISL3-like insertion sequence from *Pseudomonas stutzeri* AN10 involved in catabolic gene inactivation

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Summary. A novel insertion sequence (IS), *ISPst9*, from *Pseudomonas stutzeri* AN10 was cloned and characterized. *ISPst9* is a typical bacterial IS, consisting of a 2472-bp element flanked by 24-bp perfect inverted repeats that generates 8-bp AT-rich target duplications upon insertion. The sequence also contains a gene that encodes an active transposase (TnpA) with significant amino acid identity to members of the ISL3 family. Southern blot analysis of digested genomic DNA of strain AN10 and its 4-chlorosalicylate-degrading derivative strain AN142 demonstrated that native *ISPst9* transposes in multiple copies, with one of them responsible for the *nahH* insertional inactivation observed in strain AN142. Precise excision of *ISPst9* yielded *NahH*⁺ revertants of AN142 at high frequencies (up to 10⁻⁶). In vivo transposition, mainly in multiple copies, of an *ISPst9* derivative containing a Km^R cassette cloned into a suicide vector was also demonstrated. Hybridization experiments carried out with different strains of *P. stutzeri* and with 292 phylogenetically distinct environmental isolates suggested that the presence of an *ISPst9*-like IS occurs in diverse bacteria together with the presence of aromatic hydrocarbon-degrading determinants. [*Int Microbiol* 2008; 11(2):101-110]

Key words: *Pseudomonas stutzeri* · insertion sequences · mobile elements · transposition · catabolic gene inactivation

Introduction

Pseudomonas stutzeri AN10 is a naphthalene-degrading bacterium able to aerobically dissimilate naphthalene, 2-methylnaphthalene, and salicylate as sole carbon and energy sources [44]. As described for other *Pseudomonas*, these aromatic hydrocarbons are funneled to the Krebs cycle through catechol (and its methyl-derivatives) [15]. Naphthalene degradation genes (*nah* genes) of *P. stutzeri* AN10 are chromosomally encoded and organized in four operons: (i) the upper pathway,

coding for enzymes involved in the conversion of naphthalene to salicylate (genes *nahAaAbAcAdBFCE*); (ii) the lower pathway, encoding enzymes that convert salicylate to catechol and further to pyruvate and acetyl-CoA (genes *nahGTHINLOMKJ*); (iii) the regulatory gene *nahR*; and (iv) a second salicylate hydroxylase gene (*nahW*) [8–10]. To improve the catabolic potential of strain AN10, a derivative strain, designated *P. stutzeri* AN142, was constructed in our laboratory (Ginard M, 1997, Ph.D. thesis). Strain AN142 is a *P. stutzeri* AN10 spontaneous mutant in *nahH* (catechol 2,3-dioxygenase gene) that received the *clc* element of *Pseudomonas* sp. B13 carrying the genes comprising the chlorobenzoate-degradative pathway (*clcABDE*) [40] by conjugative transfer, thus allowing growth of the strain on 4-chlorosalicylate.

As described for many other catabolic genes [35,47], entire copies and the remnants of insertion sequence (IS) elements were found beside the *nah* determinants of strain

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AN10 [8–10]. IS elements are the simplest mobile genetic elements (usually less than 2.5 kb in length); they generally encode a protein required for transposition (transposase) and are found in the genomes of nearly all bacteria [11,32]. As traditionally described for the acquisition of antibiotic resistance [34], the presence of IS elements adjacent to catabolic genes has provided further support for the modular theory of evolution of modern catabolic pathways, suggesting that catabolic modules are recruited, assembled, and mobilized between bacteria by transposition events [10,52,55]. The *in situ* spread and even *de novo* construction of catabolic pathways in bacteria have allowed bacterial communities to rapidly adapt to the presence of xenobiotic organic compounds [reviewed in 50]. In addition to their role in gene recruitment and mobilization, IS elements of nearly all known IS families have been described as agents involved in the modulation of gene expression, either by polar mutation due to direct gene disruption, indirect reduction of expression of genes downstream of the insertion point, or by transcriptional activation of silent genes due to the presence of outwardly directed regulatory sequences [11,32]. Of special interest for this study are three well-characterized IS elements belonging to the ISL3 family: IS*1411* from the phenol-degrading *Pseudomonas* sp. strain EST1001, discovered as a result of insertional activation of promoterless phenol degradation genes (*pheBA*) [28]; IS*Pst2* of *Pseudomonas* sp. strain OX1, formerly *P. stutzeri* strain OX1 [4,13,39], which inactivates the *m*-xylene and *p*-xylene catabolic pathway as well as the *o*-xylene catabolic genes in its derivative strain M1 [7]; and IS*Ppu12* from the toluene-xylene catabolic plasmid pWW0 of *P. putida* mt-2, whose insertional inactivation in *xylE* (catechol 2,3-dioxygenase-encoding gene) allowed, after conjugative transfer of this plasmid to *Pseudomonas* sp. B13, the generation of derivative strain WR126, able to grow with 4-chlorobenzoate as unique carbon and energy source [56].

In this study we describe IS*Pst9*, a novel ISL3-like IS element found in *P. stutzeri* strain AN10, and present results demonstrating its role as an inactivation/reactivation agent of catabolic genes, its wide distribution in different bacteria, and its occurrence together with aromatic-hydrocarbon-degrading genes.

Materials and methods

Media and culture conditions. *Pseudomonas* strains were grown on minimal medium [2] supplemented with either 10 mM succinate or 5 mM salicylate (or derivatives) as required. From a beach polluted after the *Prestige* oil spill (Galicia, Spain), 292 phylogenetically distinct environmental isolates were obtained. These isolates were grown at 25°C on minimal [2] or marine media supplemented, when necessary, with 10 mM succinate. Marine medium was prepared with artificial sea water (Scharlau) buffered

with 0.1 M Tris-HCl, pH 7.4, and supplemented with 0.27 g NH₄Cl/l and 89 mg Na₂HPO₄ · 7 H₂O/l as nitrogen and phosphorus sources. An autoclaved solution of Fe(NH₄)₂(SO₄) · 6H₂O was added at a final concentration of 2 mg/l. For solid marine medium, double-strength liquid medium was prepared and mixed with an equal volume of melted, sterile, washed agar (Scharlau). *Escherichia coli* and *Klebsiella pneumoniae* strains were grown at 30°C in Luria-Bertani (LB) medium [45]. Ampicillin, kanamycin, and tetracycline were added to final concentrations of 100, 50, and 10 µg/ml, respectively, to select for the presence of plasmids. LB medium supplemented with rifampicin (40 µg/ml) was used to obtain and maintain *E. coli* strain DH5α Rif^r.

General DNA manipulations. Plasmids were purified using the QIAprep Spin Miniprep Kit (Qiagen). Genomic DNA from *Escherichia*, *Klebsiella*, and *Pseudomonas* strains were prepared by the method of Dhaese and co-workers [14]. Genomic DNA preparations from environmental isolates were done as previously described [54], with three additional freeze-thaw steps in liquid nitrogen. Restriction endonuclease digestions (Promega and GE Healthcare) and ligations with T4 DNA ligase (Invitrogen) were done as recommended by manufacturers. DNA fragments were recovered from agarose gels using the QIAquick Gel Extraction Kit (Qiagen). All other DNA manipulations were carried out according to standard procedures [45].

PCR amplification and hybridization. The location of IS*Pst9* in the *nah* cluster of *P. stutzeri* AN10 and its derivatives was routinely analyzed by PCR amplification with *Taq* DNA polymerase (GE Healthcare), using combinations of the following primers: ISMG4 (5'-TGGTCAGTGCAC-CTCGTTC-3'), SAL23 (5'-CAAGGCCCTGGAACACTACG-3'), SAL45 (5'-GGTCTTGCCGTGGGTCAGG-3'), SAL64 (5'-TCTCCAGGTTAGC-CATTGATC-3'), and SAL71 (5'-GGCACAGGCAACGGCTATTC-3'), and the amplification conditions reported previously [36].

Southern blot hybridization was done as described by Sambrook and Russell [45]. Dot-blot hybridization (1 µg of genomic DNA per blot) was carried out with a MilliBlot-D vacuum manifold (Millipore) according to the manufacturer's instructions. Enhanced chemiluminescence direct labeling (ECL Direct Nucleic Acid Labeling and Detection System, GE Healthcare) was used for hybridization. Gene-specific probes were prepared from *P. stutzeri* AN10 genomic DNA by PCR amplification with *Taq* DNA polymerase (GE Healthcare). Probes, their lengths, and the primers used to obtain them were as follows: IS*Pst9* probe, 0.64-kb, ISMG9 (5'-ATCCACG-GCAAGCGGGTC-3') and ISMG3 (5'-CTCCCGGTCTGAGACTTCG-3') primers; *nahAc* probe, 0.87-kb, AC149F and AC1014R primers [19]; *catA* probe, 0.45-kb, 1C12OF and 2C12OR primers [20]; *nahH* probe, 0.56-kb, 3C23O and 4C23O primers [12]; *nahW* probe, 0.66-kb, F-*nahW* and R-*nahW* primers [9]; *nosZ* probe, 0.47-kb, U1672 and L2140 primers [12]. The 16S rDNA probe (1.46 kb) was generated using a mixture of genomic DNAs from different environmental isolates, the primers (16S-27F and 16S-1492R), and PCR conditions reported previously [31,36]. The kanamycin resistance gene probe was generated by excision with *EcoRV* of the Km^r cassette from pCSI2 plasmid [17]. The hybridization signal in the dots was quantified using the GeneTools analysis program (SynGene). Spearman's rank correlation analysis of relative hybridization data was performed with SPSS 13.0 statistical data analysis package (SPSS).

Plasmid constructions. Plasmid pMGV01 was obtained by cloning a 4.1-kb *PstI*-*XhoI* fragment from *P. stutzeri* AN142 (Ginard M, 1997, Ph.D. thesis), containing the entire IS*Pst9*, into the *PstI* and *XhoI* sites in the multi-cloning site (MCS) of pBluescript SK (–) (Stratagene). The fragment was obtained from *P. stutzeri* AN142 genomic DNA by PCR amplification with primers SAL20 (5'-GCTGCGTCAAGGGTAAGAGG-3') and SAL25 (5'-CGGCGAACACCTTGAAATTGG-3') using the proof-reading AccuPrime *Pfx* DNA polymerase (Invitrogen), followed by amplicon digestion with both *PstI* and *XhoI*. Sequential restriction digestions of pMGV01 with *NaeI* and *PvuII*, followed in each case by self-ligations, yielded plasmid pJOC02. A novel PCR-generated *SmaI* restriction site 279 bp upstream of the putative *orfI* initiation codon (703-agcccGggtta-712 instead of 703-agcccCggtta-712,

coordinates from GenBank accession no. AJ582631) was constructed by PCR amplification of the entire pJOC02 plasmid with primers SMA1 (5'-AGCCCGGGTACAAGAAGC-3') and SMA2 (5'-TACCCGGGCTATTGTCAAG-3') using AccuPrime Pfx DNA polymerase (Invitrogen), followed by restriction with *Sma*I and self-ligation (plasmid pJOC03). Plasmid pJOC06 was obtained by cloning the 2.76-kb *Eco*RI-*Sal*I fragment from plasmid pJOC03 into the *Eco*RI and *Sal*I sites in the MCS of the suicide vector pGP704 [33]. This conjugative plasmid is stable only in *E. coli* strains harboring the replication machinery of λ phage (λ_{pir} strains). The *Eco*RI-*Sal*I fragment was obtained by PCR amplification with primers ECOJOC (5'-GAATTCTGGACATGAGCAAGGCC-3') and SALJOC (5'-GTCGACA-CAACTGTTTCAGCTCACC-3') using AccuPrime Pfx DNA polymerase (Invitrogen). Finally, the *Eco*RV-excised Km^R cassette from pCSI2 [17] was cloned into the PCR-generated *Sma*I site of pJOC06, generating plasmid pJOC09. *E. coli* strains DH5 α [25] and S17.1 λ_{pir} [26] were used as hosts in cloning experiments with pBluescript SK (-) and pGP704 derivatives, respectively.

Phenotype reversion and transposition experiments. For phenotype reversion experiments, *P. stutzeri* AN142 was grown overnight in minimal medium supplemented with 5 mM 4-chlorosalicylate. Culture and serial dilutions thereof were plated onto four sets of minimal medium agar plates containing 10 mM succinate (for counting all AN142 descendants), and 5 mM 3-, 4-, and 5-methylsalicylate (for counting *nahH*^r revertants), and the plates were incubated at 30°C for 48 h. The frequency of reversion was calculated from three independent experiments for each chemical compound as the ratio of revertants to all descendants. Three revertants (*P. stutzeri* strains R3, R4, and R5; obtained on 3-methyl-, 4-methyl-, and 5-methylsalicylate plates, respectively) were maintained for hybridization experiments.

Plasmid pJOC09 was transferred by mating from *E. coli* S17.1 λ_{pir} into three recipient strains: *P. stutzeri* AN10, a Rif^r spontaneous mutant of *E. coli* DH5 α , and *K. pneumoniae* CMD1 [51]. Aliquots of stationary-phase cultures of donor (100 μ l) and recipient (100 μ l) cells were spotted together onto the surface of a LB agar plate and incubated at 30°C for 6 h. Afterwards, the cell mixture was re-suspended in 1 ml of Ringer solution (Merck). This mixture was serially diluted and plated onto mineral medium agar plates containing succinate plus kanamycin (for transconjugants), or succinate alone (for all recipients), and the plates were incubated at 30°C for 48 h. The frequency of conjugation plus transposition for each strain was calculated from three independent experiments as the ratio of transconjugants to all recipients.

DNA sequencing and analysis. Nucleotide sequences were determined (both strands) directly from plasmids pMGV01 and pPA50-C [10] using the BigDye terminator cycle sequencing kit (Applied Biosystems) according to the manufacturer's instructions. Sequences were extended by primer-walking, with the design of new primers based on the sequences determined in this study. Primer design and sequence analyses were done using BioEdit 6.0.5 sequence alignment editor [24], Vector NTI 10.0 suite (Invitrogen), and EMBOSS suite [42]. Similarity searches with GenBank and EMBL databases were done using BLASTP (NCBI) and FASTA-protein (EBI) web tools, respectively [1,38]. The ClustalX program [48] was used for amino acid sequence alignments. The ProtDist program of the PHYLIP 3.6 package [18] and PhyML program [22] were used for inferring distance- and likelihood-based phylogenies, respectively. Kimura's distance, Dayhoff PAM matrix, and the Jones-Taylor-Thornton model were used as models of amino acid substitution in both approaches. Bootstrap (100 replicates) was undertaken to construct distance-based trees using the neighbor-joining method. Resulting phylogenies were displayed using the TreeView 1.6.6 program [37].

Nucleotide sequences accession numbers. The sequence of ISPst9, as determined from the insert in pMGV01, was deposited in GenBank under accession no. AJ582631. The GenBank accession no. for the sequence of the *P. stutzeri* AN10 ISPst9 insertion sequence and its flanking region is DQ473406.

Results

Isolation and characterization of ISPst9. ISPst9 was discovered during an analysis of the *nahH* (catechol 2,3-dioxygenase-encoding gene) mutation present in *P. stutzeri* AN142, the 4-chlorosalicylate-degrading derivative of *P. stutzeri* AN10. A *Pst*II-*Xho*I-digested chromosomal DNA of AN142 and the parental *P. stutzeri* strain AN10 were subjected to electrophoresis and Southern blot hybridization against a *nahH* probe generated by PCR from strain AN10. The results indicated that *P. stutzeri* AN142 had acquired a 2.5-kb DNA insertion in *nahH* (Fig. 1A). After PCR amplification of the insertion with primers SAL20 and SAL25 (a schematic representation of their location on *P. stutzeri* AN142 genome is shown in Fig. 1B) and cloning of the resulting fragment to generate plasmid pMGV01, the complete nucleotide sequence of the DNA insertion on *nahH* in *P. stutzeri* AN142 was determined. Sequence analysis revealed that the DNA insertion was 2472 bp in length and occurred after nucleotide 159 of *nahH*. The DNA insertion was flanked by an 8-bp direct repeat of *nahH* bases 152–159 (5'-ACAAATTC-3'). It had two perfect 24-bp inverted repeats at either end (5'-GGGT-ATGCGGATTTAATGGTTGAT-3') and coded for a putative transposase (see below). Thus, as it contained the main features of an insertion sequence, the insert was named ISPst9, accordingly to the IS database [<http://www-is.biotoul.fr/is.html>].

Computer-aided analysis of the ISPst9 DNA sequence indicated the presence of two potential open-reading frames (ORFs) transcribed in the same direction (Fig. 1B). The first ORF (*orf1*) coded for a putative protein of 206 amino acids with a predicted mass of 21.8 kDa. The amino acid sequence analysis indicated that this protein belongs to a LysE-type translocator protein family (pfam01810) as it showed the highest amino acid identity values (41–43%) with LysE-like proteins from whole-genome sequences of *Pseudomonas* species. The second ORF (*tnpA4*) coded for a putative protein of 429 amino acids with a predicted mass of 50.3 kDa. The deduced amino acid sequence of *tnpA4* (TnpA4) showed the highest level of homology with two transposase-like putative proteins annotated from the whole-genome sequence of *Idiomarina loihiensis* (FASTA amino acid identity: 93 and 92%, in 429 amino acids, with Q5QX23 and Q5QUL5, respectively) [27]. In addition, TnpA4 was highly homologous to two well-characterized ISL3-family transposases from hydrocarbon-degrading *Pseudomonas* strains (FASTA amino acid identity: 93% in 404 amino acids with O52212, the ISPpu12 transposase from *P. putida* mt-2, and 87% in 423 amino acids with Q9X7J2, the ISPst2 transposase from *Pseudomonas* sp. OX1) [7,53,56]. Multiple alignments based on these and other ISL3-like transposases retrieved from the IS database were conducted. The results showed that the

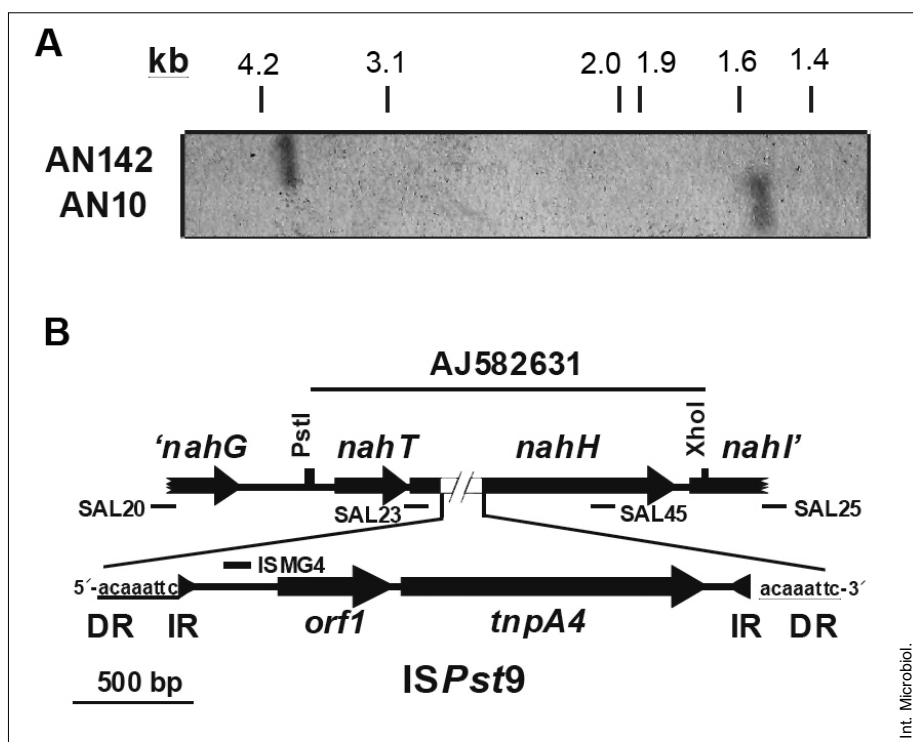


Fig. 1. Location of ISPst9 in *Pseudomonas stutzeri* strain AN142. **(A)** Southern blot of *P. stutzeri* AN142 and AN10 genomic DNAs digested with *Pst*I and *Xho*I and probed with the *nahH* probe. **(B)** Schematic representation of ISPst9 in *P. stutzeri* AN142 and its flanking regions. Genes code for the following proteins: *nahG*, salicylate hydroxylase; *nahH*, catechol 2,3-dioxygenase; *nahI*, hydroxymuconic semialdehyde dehydrogenase; *nahT*, ferredoxin-like protein; *orf1*, LysE-like protein; *tnpA4*, transposase-like protein. Arrows indicate the directions of gene transcriptions. ISMG4, SAL20, SAL23, SAL25, and SAL45 denote the primers used. Thin lines indicate their location and 5'–3' orientation. IR and DR denote inverted and directed repeats of ISPst9, respectively. Line below AJ582631 accession number indicates sequence data submitted to GenBank.

sequence of TnpA4 had the conserved DDE motif of active transposases [11]. Phylogenetic trees were generated for TnpA4 and homologous transposases retrieved from the IS database (> 40% amino acid identity). All distance-based and maximum-likelihood phylogenetic approaches showed, independent of the amino acid substitution model used, that the sequence of ISPst9 transposase TnpA4 grouped with ISL3 transposases from environmental gamma-proteobacterial isolates, mainly aromatic-hydrocarbon-degrading bacteria (Fig. 2, group 1), and was clearly distinct from those in group 2, mainly constituted by ISL3-like insertion sequences identified in clinical isolates.

Location and number of ISPst9 in the genome of *P. stutzeri* AN10 and its derivative *P. stutzeri* AN142. Sequence analysis of ISPst9 from *P. stutzeri* AN142 revealed that 94 nucleotides at its 3'-end, downstream of *tnpA4*, overlapped with 100% identity with the 5'-end of the sequence of a genome fragment from *P. stutzeri* AN10, which contains the regulatory- (*nahR*) and catabolic-salicylate-degrading (*nahW* and *nahGTHINLOMKJ*) genes as determined previously (sequence AF039534 [9,10]). This result suggested that a copy of ISPst9 was originally located beside salicylate-degrading genes in strain AN10. To prove this, we sequenced the nucleotides located upstream *tnpA3* (an IS5-like transposase-encoding gene) of *P. stutzeri* AN10, which was already known [10]. Sequence analysis confirmed the presence in *P. stutzeri* AN10 of an identical copy of ISPst9

(100% nucleotide identity) upstream of *tnpA3* (Fig. 3A). Computer-aided analysis also showed the presence of two partial ('*orf2* and '*orf4*') and one complete ORF (*orf3*) located upstream of ISPst9 in *P. stutzeri* AN10 (Fig. 3A). The '*orf2*' partial C-terminal gene product (153 amino acids) was homologous to the C-terminal domain of acetoacetyl-CoA synthases from several *Pseudomonas* strains (amino acid identity values: 73.8% to Q9I2B2 of *P. aeruginosa* PAO1, 75.7% to Q4K4Q4 of *P. fluorescens* Pf-5, and 71.0% to Q88IC8 of *P. putida* KT2440). The '*orf3*' gene product (330 amino acids) resembled a lactone hydrolase (62.4% amino acid identity to the CamQ lactone hydrolase of pCAM plasmid from *P. putida* NCIMB 10007, Q6STL9). Finally, the '*orf4*' partial C-terminal gene product (96 amino acids) showed 62.8% identity to the C-terminal domain of the CamR transcriptional repressor encoded on pCAM plasmid from *P. putida* PpG1, Q6STL9 [3].

To determine whether the original copy of ISPst9 present in *P. stutzeri* AN10 was maintained in *P. stutzeri* AN142 after the transposition event, shown in Fig. 1A, a set of PCR reactions was developed to analyze the location of ISPst9 in *P. stutzeri* AN142. The presence of ISPst9 upstream of *tnpA3* was evaluated with primers SAL71, SAL64, and ISMG4 (a schematic representation of their location on *P. stutzeri* AN10 genome is shown in Fig. 3A); inactivation of *nahH* by ISPst9 in *P. stutzeri* AN142 was confirmed using primers SAL23, SAL45, and ISMG4 (a schematic representation of their location is shown in Fig. 1B). As shown in Fig. 3B, PCR amplifications using the

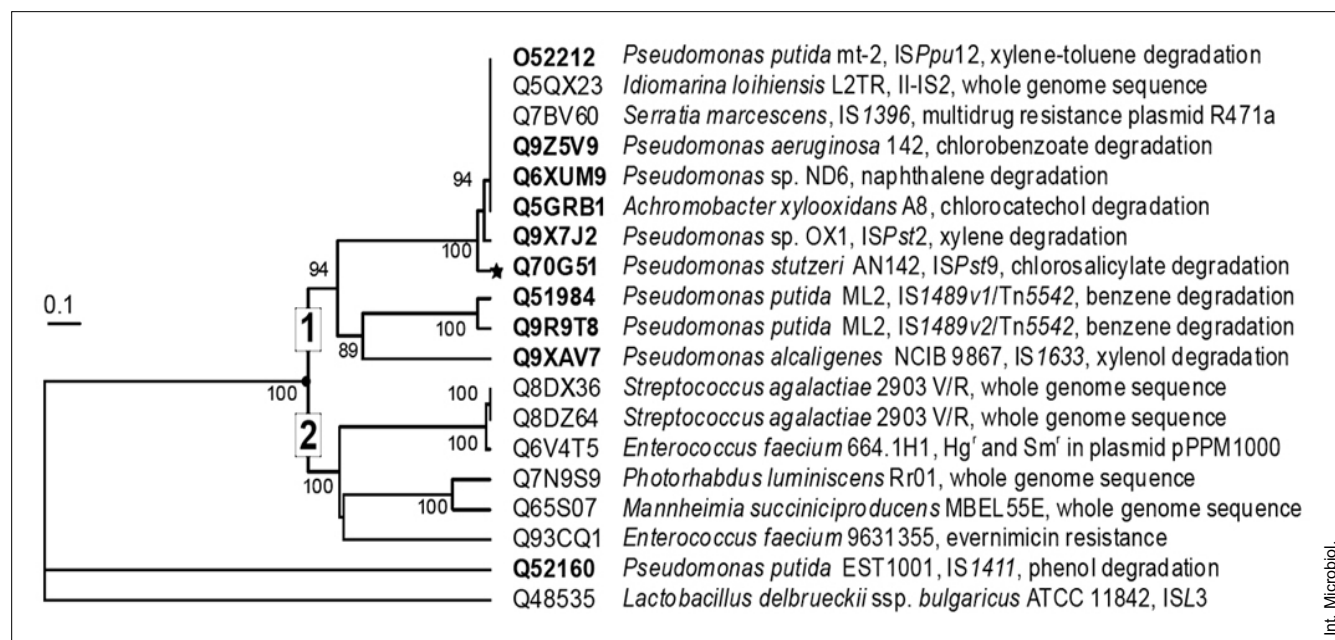


Fig. 2. Phylogenetic relationships between TnpA4 of IS*Ps9* and its closest relatives. The neighbor-joining tree is a distance-based phylogeny using Kimura's distance model. Accession numbers in bold indicate the transposases involved and/or related with aromatic hydrocarbon degradation. Star indicates the TnpA4 amino acid sequence. Numbers in nodes are Bootstrap values (>85). Transposases of ISL3 from *Lactobacillus delbrueckii* ssp. *bulgaricus* ATCC 11842 (Q48535) and IS1411 from the phenol-degrading strain *P. putida* EST1001 (Q52160) were used as outgroup sequences.

SAL71 and SAL64 primers and the SAL71 and ISMG4 primers produced single bands of 3.5-kb and 0.8-kb, respectively, for strains AN10 and AN142. Thus, the original copy of *ISPst9* in *P. stutzeri* AN10 was maintained upstream of *tnpA3* in *P. stutzeri* AN142. PCR amplifications with the SAL23 and SAL45 primers and the SAL23 and ISMG4 primers corroborated that *nahH* of *P. stutzeri* AN142 was inactivated by *ISPst9* but was intact in *P. stutzeri* AN10. To analyze whether more than two copies of *ISPst9* were present in the *P. stutzeri* AN142 genome, Southern blot hybridizations against *Eco*RI-digested genomic DNAs from strains AN10 and AN142 were done using probes against *ISPst9* and *nahH* genes. The results revealed the presence of a unique copy of *ISPst9* in *P. stutzeri* AN10 while strain AN142 had, at least, eight copies of *ISPst9*, including the original one (Fig. 3C). Southern blot hybridization also confirmed the presence of a unique copy of *nahH* in strain AN142, which hybridized also with a copy previously detected for *ISPst9* (indicated with an arrow in Fig. 3C). No hybridization signal was observed in *Pseudomonas* sp. B13 (result not shown), thus suggesting that all new *ISPst9* copies observed in strain AN142 came from the one detected in *P. stutzeri* AN10.

Reversion of *P. stutzeri* AN142 to a NahH⁺ phenotype. Although *P. stutzeri* AN142 was able to grow on 4-chlorosalicylate because it carried the *clc* element from *Pseudomonas* sp. B13 [40], this strain was unable to grow on

methylsalicylates. This is because ortho-cleavage of methylcatechols by the catechol 1,2-dioxygenase encoded in plasmid B13 yields methylmuconolactones [49], which are dead-end metabolites. Thus, growth of strain AN142 on methylsalicylates was only possible through the recovery of methylcatechols meta-cleavage activity mediated by NahH (catechol 2,3-dioxygenase); this would imply a precise excision of *ISPst9* from the *nahH* gene of *P. stutzeri* AN142. Putative NahH⁺ revertants able to use three different methylsalicylates were isolated by plating cultures of strain AN142, grown on 4-chlorosalicylate, on minimal medium supplemented with 3-, 4-, or 5-methylsalicylate as unique carbon and energy sources. Similar reversion frequencies were observed in all cases ($1.4 \pm 0.4 \times 10^{-6}$ on 3-methylsalicylate, $8.8 \pm 0.6 \times 10^{-6}$ on 4-methylsalicylate, and $4.6 \pm 0.5 \times 10^{-6}$ on 5-methylsalicylate). Three revertants (designated as R3, R4, and R5; from 3-, 4-, and 5-methylsalicylate plates, respectively), from each of the isolation strategies, were selected for further analysis. PCR analysis showed that all revertants maintained the *ISPst9* copy upstream of *tnpA3*, the original position in AN10, and that they had lost the *ISPst9* copy interrupting *nahH* (results not shown). Furthermore, Southern blot hybridizations against *Eco*RI-digested genomic DNAs from all three revertants confirmed the loss of only one copy of *ISPst9*, that interrupted *nahH* (indicated with an arrow in Fig. 3C), and suggested the recovery of the original *nahH* gene structure. To clarify how the excision of *ISPst9* restored

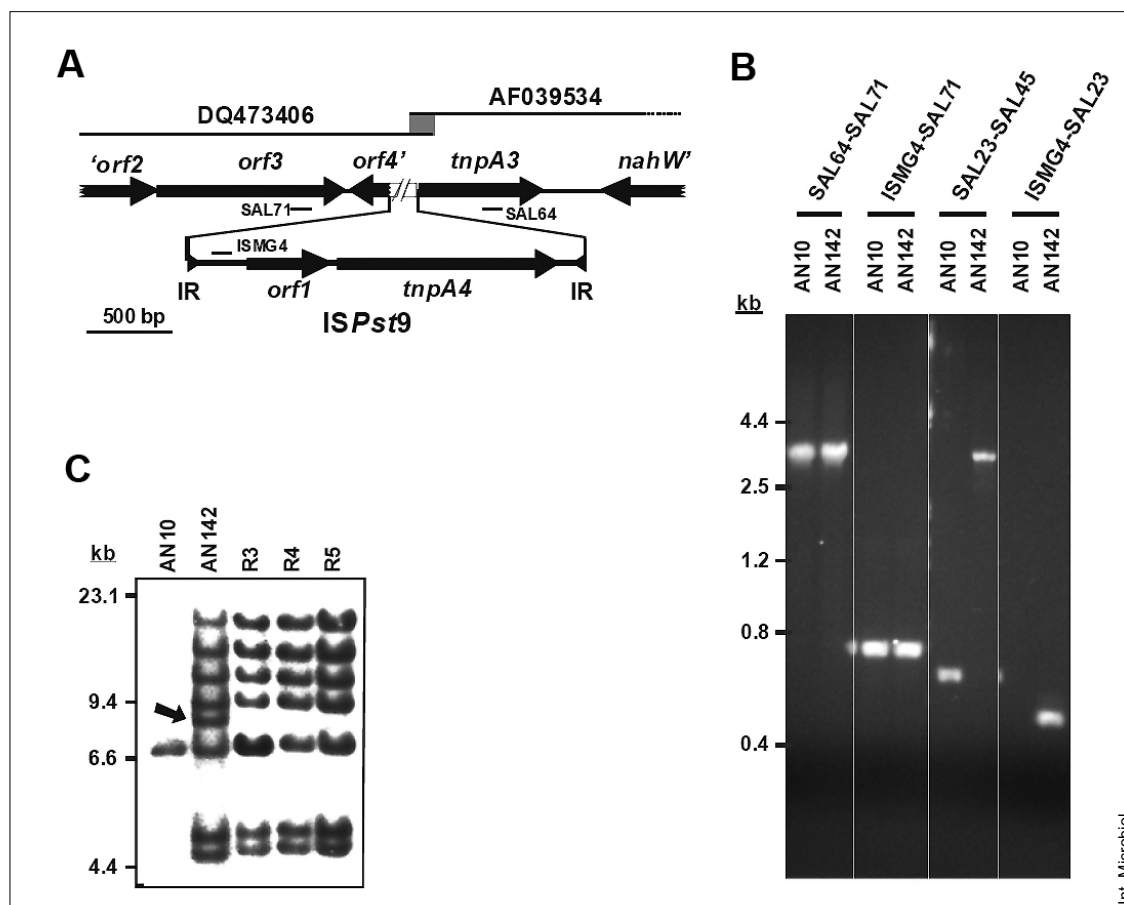


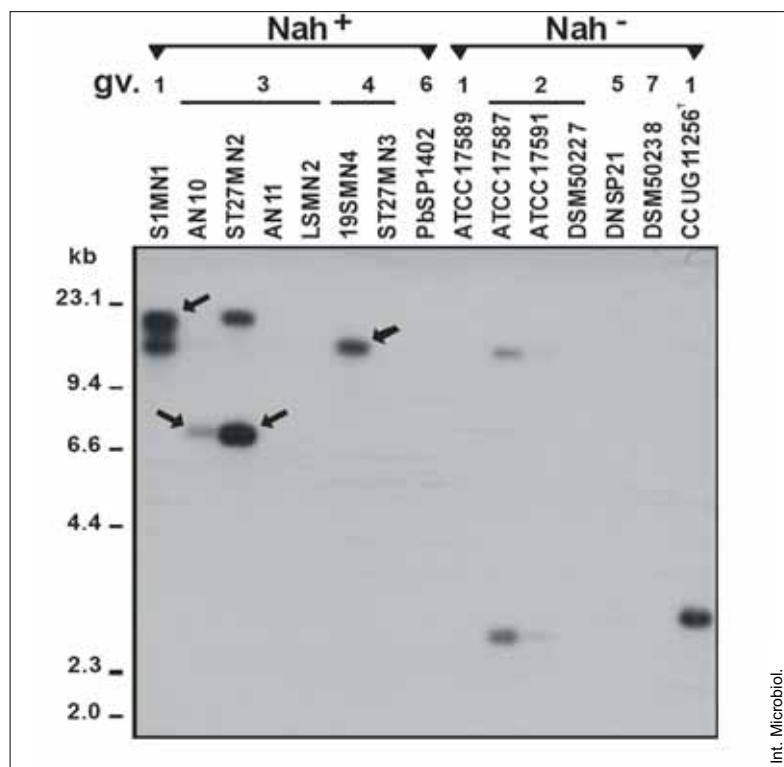
Fig. 3. Location of *ISPst9* in *P. stutzeri* AN10. **(A)** Schematic representation of *ISPst9* in *Pseudomonas stutzeri* AN10 and its flanking regions. Genes code for the following proteins: *nahW*, salicylate hydroxylase; *orf1*, LysE-like protein; *orf2*, partial acetoacetyl-CoA-synthase-like protein; *orf3*, CamQ lactone-hydrolase-like protein; *orf4*, partial CamR transcriptional regulator. Arrows indicate the directions of gene transcriptions. ISMG4, SAL64, and SAL71 denote primers used. Thin lines indicate their location and 5'-3' orientation. IR denotes inverted repeats of *ISPst9*. Lines below AF039534 and DQ473406 accession numbers indicate sequence data submitted to GenBank. **(B)** Agarose gel showing the PCR products obtained for *P. stutzeri* strains AN10 and AN142 using the primers indicated. Locations and 5'-3' orientations of primers are shown in Figs. 1B and 3A. **(C)** Southern blot hybridization of *P. stutzeri* AN10, AN142, R3, R4, and R5 genomic DNAs digested with *Eco*RI and hybridized with the *ISPst9* probe. Arrow indicates the *Eco*RI fragment containing the *ISPst9* copy that interrupts *nahH* in strain AN142.

NahH function, the *nahTH* region of all three revertants (R3, R4, and R5) was amplified using SAL23 and SAL45 primers (a schematic representation of their location is shown in Fig. 1B) and sequenced. The *nahH* sequences of all three revertants were identical to that of strain AN10, not conserving the 5'-ACAAATTC-3' direct repeat sequence duplicated in the insertion event in strain AN142. This result confirmed the precise excision of *ISPst9* from *nahH*.

Demonstration of *ISPst9* transposition in other bacteria. To demonstrate that *ISPst9* was able to transpose, plasmid pJOC09, carrying *ISPst9* with an inserted Km^R cassette as phenotypic marker, was constructed as described in Materials and methods. In pJOC09, the Km^R cassette was inserted in a PCR-generated *Sma*I restriction site

located 279 bp upstream of the ATG codon of *orf1* in *ISPst9*, as it was thought that, in this location, the cassette would not disable any transposition function by affecting the expression of the *ISPst9* genes. As pJOC09 plasmid is a derivative of the suicide vector pGP704 [33], this plasmid is stable only in λ_{pir} derivatives of *E. coli* and can be transferred from those strains into recipient strains but will not replicate within them. Thus, conjugation experiments between *E. coli* S17.1 λ_{pir} carrying pJOC09 with strains of distinct bacterial species (*K. pneumoniae* CMD1, *E. coli* DH5 α Rif^R, and *P. stutzeri* AN10) were done. In all mating experiments, a significant number of Km^R transconjugants with a wide range of transposition frequencies were obtained ($9.6 \pm 1.1 \times 10^{-5}$ for *P. stutzeri*, $9.3 \pm 0.9 \times 10^{-6}$ for *E. coli*, and $7.8 \pm 6.2 \times 10^{-8}$ for *K. pneumoniae*). Southern blot hybridization of chromosomal digests of transconjugants

Fig. 4. Southern blot hybridization with the *ISPst9* probe of *Eco*RI-digested genomic DNAs from several naphthalene-degrading (Nah^+) and non-degrading (Nah^-) strains of *Pseudomonas stutzeri* that belong to different genomovars (gv.). Strain sources are as follows: ATCC17587, ATCC17589, and CCUG11256^T [46]; 19SMN4, AN10, DNSP21, DSM50227, and DSM50238 [43]; AN11, LSMN2, S1MN1, ST27MN2, and ST27MN3 [21]. *P. balearica* strain SP1402^T (formerly *P. stutzeri*, PbSP1402) was also included [5]. Arrows indicate those *Eco*RI DNA fragments that hybridized with the *nahW* probe.



against a Km^R cassette probe revealed that most isolates harbored more than one copy of the *ISPst9* derivatives, as expected, with six being the highest number of copies detected (results not shown).

ISPst9 in genomes of *P. stutzeri* strains and other bacteria. As noted above, *ISPst9* belongs to a sub-family of *ISL3*-like insertion sequences found mainly in aromatic-hydrocarbon-degrading bacteria (group 1 in Fig. 2): therefore, their presence in a genome may be related to the occurrence of genes encoding proteins with this function. To evaluate the simultaneous occurrence of both *ISPst9* and *nah* genes, Southern blot hybridizations against *Eco*RI-digested genomic DNAs from several naphthalene-degrading (7 isolates) and non-degrading (7 isolates) strains of *P. stutzeri* from six different genomovars were done using a probe for *ISPst9* from strain AN10 (Fig. 4). The naphthalene-degrading *P. balearica* (formerly *P. stutzeri* gv. 6) strain SP1402^T [5] was also included in this experiment. Representatives of four *P. stutzeri* genomovars (gvs. 1–4) had one or more copies of an *ISPst9*-like insertion sequence in their genomes. *ISPst9*-like insertion sequences were detected in half of the naphthalene-degrading strains and in one-third of the non-degrading strains, suggesting that the occurrence of *ISPst9*-like insertion sequences in *P. stutzeri* was not restricted to naphthalene-degrading isolates, although it was more frequent in them. Moreover, the size of one *ISPst9* fragment of each

naphthalene-degrading *P. stutzeri* strain was similar to the one that hybridized with an internal probe for the gene *nahW* (salicylate hydroxylase) (Fig. 4). This suggested that the two genetic elements (*ISPst9* and *nahW*), if coexistent, occurred together, as shown for strain AN10. Interestingly, all of the Nah^+ but none of the Nah^- *P. stutzeri* isolates hybridized with the *nahW* probe (result not shown).

To extend this study to other bacteria, 292 environmental isolates obtained from samples polluted by *Prestige* tanker fuel that were collected at a beach in Galicia, NE Spain, and from non-polluted samples collected at the same beach were used. The phylogenetic affiliations of the isolates were determined by 16S rDNA analyses (data not shown), which revealed that 57% belonged to gammaproteobacteria (167 isolates). Of these, 33% were identified as *Pseudomonas* (97 isolates) and 13% as *P. stutzeri* (39 isolates). Dot-blot hybridizations against genomic DNA were done using DNA probes for *ISPst9* and five other selected genes: 16S rDNA, used as the control gene; *nahAc* (naphthalene 1,2-dioxygenase α -subunit) and *nahH* (catechol 2,3-dioxygenase), two widespread naphthalene-catabolizing genes present in *Pseudomonas* and non-*Pseudomonas* naphthalene-degrading strains [10,23, 29]; and *nosZ* (nitrous oxide reductase) and *cata* (catechol 1,2-dioxygenase), two genes considered as characteristic for *P. stutzeri* species and used as phylogenetic markers for this species [30]. Hybridization experiments were followed by correlation analysis of the relative

Table 1. Spearman's rank correlation (r) of hybridization data obtained with environmental isolates*

Probes used	All (n = 292)	Gammaproteobacteria (n = 169)	<i>Pseudomonas</i> sp. (n = 97)	<i>Pseudomonas stutzeri</i> (n = 39)
ISPst9 vs <i>nahH</i>	0.75	0.77	0.64	0.80
ISPst9 vs <i>nahAc</i>	0.78	0.76	0.75	0.87
ISPst9 vs 16S rDNA	0.37	0.32	0.62	0.39*
<i>nahAc</i> vs <i>nahH</i>	0.84	0.85	0.88	0.88
<i>nosZ</i> vs <i>catA</i>	0.63	0.69	0.56	0.79

P for each r value is <0.001 , with the exception of ISPst9 vs 16S rDNA in *P. stutzeri*, whose P is 0.014.

hybridization intensities for the different genes analyzed (Table 1). As expected, high correlation values ($r = 0.84$ – 0.88) were observed in the presence of both naphthalene degradation genes (*nahAc* and *nahH*), independent of the phylogenetic group analyzed. The correlation between the hybridization values obtained for ISPst9 and 16S rDNA probes was lower ($r = 0.37$ – 0.62), as expected. Note that the correlation values between the presence of both *nosZ* and *catA* genes were highest only when *P. stutzeri* isolates were considered ($r = 0.79$). The values decreased when isolates from other species were added to the analysis ($r = 0.56$ – 0.69), confirming the importance of these two genes in the definition of *P. stutzeri* species. Correlation values between ISPst9 and the naphthalene-catabolizing genes *nahAc* and *nahH* were high (*nahH*, $r = 0.64$ – 0.80 ; *nahAc*, $r = 0.75$ – 0.87), i.e., similar to those measured for the genes thought to coexist, such as *nahAc* and *nahH*.

Discussion

This report describes a novel IS element of *P. stutzeri*, ISPst9, located adjacent to the naphthalene lower-degradation pathway of strain AN10. Phylogenetic comparisons of the ISPst9 transposase (TnpA4) with its closer relatives showed that ISPst9 belongs to a subfamily of ISL3-like ISs that are widespread mainly in environmental *Proteobacteria* and that it is clearly distinct from other closely related relatives found in clinical isolates. Nearly all members of the ISPst9 subfamily of ISL3-like ISs are present in aromatic-hydrocarbon-degrading bacteria. Two of them, ISPst2 from *Pseudomonas* sp. OX1 [7] and ISPpu12 of *P. putida* KT2440 [56], are involved in catabolic gene inactivation. This is also the case of ISPst9, which was first detected as a DNA insertion in *nahH* of *P. stutzeri* AN142, a 4-chlorosalicylate-degrading AN10-derivative strain constructed in our laboratory (Ginard M, 1997, Ph.D. thesis). The insertion in *nahH* inactivates meta-cleavage of 4-chlorocatechol. This step is a

biochemical prerequisite to the proper channeling of 4-chlorocatechol to intermediates of the tricarboxylic acid cycle through an ortho-cleavage pathway [41]. Note that exactly the same genotype (i.e., inactivation of the catechol 2,3-dioxygenase gene *xylE* by ISPst9-like ISPpu12) has been reported to occur in *Pseudomonas* sp. WR26 [56]. Similar behavior was also observed for ISPst2 in *Pseudomonas* sp. OX1 and in its derivative *Pseudomonas* sp. M1 [7]. In strain OX1, one copy of ISPst2 is situated upstream of *xylMA* (xylene-monooxygenase-encoding genes), between *xylW* (unknown function) and *xylC* (benzaldehyde-dehydrogenase-encoding gene), which prevents the growth of this strain on *m*-xylene and *p*-xylene by a polar effect on *xylUWCMABN* expression [7]. Alternatively, strain OX1 is able to grow on *o*-xylene as unique carbon and energy source using the *touABCDE* (toluene monooxygenase operon) gene products [6]. The *m*-xylene and *p*-xylene cometabolism by toluene monooxygenase in strain OX1 generates unproductive-growth intermediates [6]. *Pseudomonas* sp. M1 had a recovered *xylUWCMABN* genotype and showed an ISPst2 insertion in the *touA* gene of the toluene monooxygenase operon, allowing its growth on *m*- and *p*-xylene but not on *o*-xylene. In addition, the original ISPst9 copy of *P. stutzeri* AN10, located beside gene *nahW*, encoding a salicylate-degrading ability, disrupted a gene plausibly encoding CamR, the transcriptional repressor of the operon for camphor catabolism. Sequence evidence obtained in this study suggested that *P. stutzeri* AN10 harbors at least part of the genetic determinants for camphor degradation, although this strain does not grow on camphor. Thus, it could be that these genes encoding camphor degradation are a remnant of a former catabolic capability of *P. stutzeri* AN10, which might have been misregulated by the insertion of ISPst9 in the *camR* gene.

In ISPst9, ISPpu12, and ISPst2, the ISs showed the ability to transpose in multiple copies and, at least in the case of the disrupted catabolic genes, an 8-bp direct repeat was always generated [7,56]. Moreover, M1 revertants, in which ISPst2 was precisely excised from the *touABCDE* operon,

were obtained at high frequencies (10^{-6} to 10^{-3}) when *o*-xylene was used as unique carbon and energy source [7,16]. *ISPst9* had exactly the same behavior, although at lower frequencies (10^{-6}), when *P. stutzeri* AN142 was grown on methylsalicylates as unique carbon and energy sources. In both cases (*ISPst9* and *ISPst2*), only the IS copy that inactivated the catabolic gene was lost in the revertants, but there were no other changes in the IS patterns. It is difficult to explain how transposition could be the only mechanism responsible for the precise excision observed in all of the revertants analyzed. For example, it is also plausible that a recombination event between the transposition-generated direct repeats resulted in the regeneration of the disrupted catabolic genes. Thus, members of the *ISPst9* subfamily of ISL3-like ISs could act, by transposition, as inactivation agents of catabolic gene expression. Furthermore, *ISPst9*-like ISs might be precisely excised, probably helped by the recombination machinery, thus allowing the synthesis of active catabolic enzymes as needed in the respective environments. In any case, as indicated by the existence of *IS1411* [28], which is involved in transcriptional activation of genes encoding phenol degradation in *P. putida* EST1001, other ISL3-like ISs, not belonging to the *ISPst9* subfamily, are involved in catabolic gene regulation.

Using a suicide vector, we demonstrated that *ISPst9* transposes at high frequencies (up to 10^{-5} Km^R transconjugants per donor) and in multiple copies, independent of the genetic background (*P. stutzeri*, *E. coli*, and *K. pneumoniae*). These results are similar to those obtained for *ISPpu12* of *P. putida* mt-2 [56], one of its closest well-studied relatives. It was demonstrated that two identical copies of *ISPpu12* are involved in mobilization by transposition of the dehalogenase genetic determinants (*dehI* and its cognate regulatory gene, *dehR*) in the DEH element in *P. putida* PP3 [53]. Thus, experimental and sequence evidence indicates that members of the *ISPst9* subfamily of ISL3-like ISs are also involved in mobilizing catabolic determinants.

Both events, transcriptional regulation and mobilization of catabolic genes, suggest that *ISPst9*-like ISs and catabolic genes occur together in the same genome. Southern blot hybridization using an internal *ISPst9*-probe against *EcoRI*-digested genomic DNA of several *P. stutzeri* isolates revealed that the occurrence in this species of *ISPst9*-like ISs was not strictly dependent but more frequent in the presence of genes encoding naphthalene degradation. In fact, at least one copy of *ISPst9*, if present in a naphthalene-degrading *P. stutzeri* strain, could be located within the same *EcoRI*-*EcoRI* DNA fragment as *nahW* (encoding salicylate hydroxylase) in *P. stutzeri* AN10. Furthermore, dot-blot hybridization analyses done in this study with 292 phylogenetically distinct environmental isolates revealed statistically significant correlations between the occurrence of both *ISPst9* and the ana-

lyzed catabolic genes, regardless of the phylogenetic affiliation of the isolates. Our results strongly suggest that *ISPst9*-like ISs are widespread in bacteria, not only in *Gammaproteobacteria*, as previously suggested by sequence evidence [56]; and that, statistically, their presence occurs together with that of genes encoding aromatic hydrocarbon degradation or, at least, the *nah* determinants thereof.

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