Characterization of the *Pseudomonas putida* Mobile Genetic Element IS*Ppu10*: an Occupant of Repetitive Extragenic Palindromic Sequences

María Isabel Ramos-González,* María Jesús Campos, Juan Luis Ramos,
and Manuel Espinosa-Urgel

Department of Plant Biochemistry and Molecular and Cellular Biology, Estación Experimental del Zaidín,
CSIC, Profesor Albareda 1, Granada 18008, Spain

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We have characterized the *Pseudomonas putida* KT2440 insertion element IS*Ppu10*. This insertion sequence encodes a transposase which exhibits homology to the transposases and specific recombinases of the Piv/Moov family, and no inverted repeats are present at the borders of its left and right ends, thus constituting a new member of the atypical IS110/IS492 family. IS*Ppu10* was found in at least seven identical loci in the KT2440 genome, and variants were identified having an extra insertion at distinct loci. IS*Ppu10* always appeared within the core of specific repetitive extragenic palindromic (REP) sequences TCGCGGGTAAACCCGCTCCTAC, exhibiting high target stringency. One intragenic target was found associated with the truncation of a GGD EF/EAL domain protein. After active in vitro transposition to a plasmid-borne target, a duplication of the CT (underlined above) at the junction as a consequence of the IS*Ppu10* insertion was experimentally demonstrated for the first time in the IS110/IS492 family. The same duplication was observed after transposition of IS*Ppu10* from a plasmid to the chromosome of *P. putida* DOT-T1E, an IS*Ppu10*-free strain with REPs similar to those of strain KT2440. Plasmid IS*Ppu10*-mediated rearrangements were observed in vivo under laboratory conditions and in the plant rhizosphere.

Chromosomal rearrangements are a source of genetic variability on which selection can act and, although preponderantly deleterious, may produce beneficial mutations that are necessary for adaptive evolution, thus increasing bacterial fitness (19). Transposable elements (TEs) can mediate various genomic rearrangements more efficiently and often more specifically than other processes through two mechanisms that require repetitive sequences: homologous recombination and alternative transposition (see reference 20 for a review). TEs are classified by their sequence structure and transposition mechanisms. Class II TE transpose by a DNA intermediate catalyzed by a transposase. This group comprises insertion sequences (IS). IS elements contain a transposase and left and right ends, generally bounded by terminal inverted repeats, in addition to sequences that differentiate the two ends and are necessary for transposition. Although IS elements may sometimes function as genomic parasites, microbial evolution experiments demonstrate that IS elements can also promote adaptation by generating beneficial mutations (33).

In a genetic screen for functions involved in the colonization of corn seeds by *Pseudomonas putida* KT2440, Mus-9, a putative transposase/site-specific recombinase was identified (15). In this work, we demonstrate the transposase activity of this protein and present the characterization of the mobile genetic element of which it is part, IS*Ppu10*, a new member of the atypical family of insertion elements IS110/IS492. Nelson and

coworkers (28) observed that IS*Ppu10* was inserted in the repetitive extragenic palindromic (REP) sequence of *P. putida* KT2440. REPs were first detected in *Escherichia coli* and in other *Enterobacteriaceae* (35, 5) and more recently have been characterized in the soil bacterium *P. putida* (3) and detected in the genomes of some human and plant pathogens (37). It seems that REPs might be involved in important functions related to RNA and DNA physiology, although their precise function remains unclear (23, 29). IS*Ppu10* is the first IS element inserted specifically into REPs to be characterized, aside from those in enteric bacteria. The generation of a CT duplication upon insertion, which has been reported not to take place in most IS110/IS492 family elements, is also experimentally demonstrated.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and solutions. Isolation and further distribution of the efficient root colonizer and paradigm in biodegradation *P. putida* KT2440 have been recently reviewed (27). Variants of *P. putida* KT2440 from two different laboratories, Ramos' group in Granada (Spain) and Molin's group in Lyngby (Denmark), were used to compare their *mus-9* copy profiles. Other *Pseudomonas* strains were obtained from the *Pseudomonas* Reference Culture Collection (<http://artemisa.eez.csic.es/prcc>). *E. coli* DH5 α was used for cloning procedures (21), and *E. coli* HB101(pRK600) (17) was used as a helper strain in triparental mating. *E. coli* strains were grown at 37°C in Luria-Bertani (LB) medium (31). *P. putida* strains were grown at 30°C in either LB or minimal medium, which was basal M9 medium (31) supplemented with Fe-citrate (6 μ g/liter), MgSO₄ (1 mM), and trace metals as described before (1) and with sodium benzoate salt (5 mM) as the carbon source, unless otherwise specified. When appropriate, antibiotics were added to the media at the following concentrations: ampicillin, 100 μ g/ml; chloramphenicol, 30 μ g/ml; gentamicin, 10 and 100 μ g/ml for *E. coli* and *Pseudomonas* strains, respectively; kanamycin, 25 and 50 μ g/ml for *E. coli* and *Pseudomonas* strains, respectively.

Triparental filter mating. Cultures (0.5 ml) of donor, helper, and recipient bacteria grown overnight were mixed, centrifuged (12,000 rpm, 2 min), washed

* Corresponding author. Mailing address: Department of Plant Biochemistry and Molecular and Cellular Biology, Estación Experimental del Zaidín, CSIC, Profesor Albareda 1, Granada 18008, Spain. Phone: (34) 958-181600. Fax: (34) 958-129600. E-mail: maribel.ramos@eez.csic.es.

with 1 ml of LB medium, and resuspended in 25 µl of LB medium. The cells were spotted onto a nitrocellulose filter (pore size, 0.2 µm) that was placed on the surface of an LB agar plate. Controls consisting of parental strains that were not mixed were always included and incubated under similar conditions. After 16 h at 30°C, the cells were suspended in 2 ml of M9 buffer, serially diluted, and plated on selective medium for transconjugants, thus counterselecting donor and helper strains. Cells were also plated on media that permitted growth of recipients and transconjugants in order to determine the frequency of exconjugants per recipient.

Molecular biology techniques. Plasmid DNA was isolated with the QIAGEN miniprep kit. Cosmid isolation was carried out by the alkaline lysis procedure (31). Preparation of chromosomal DNA, digestion with restriction enzymes, dephosphorylation, ligation, and electrophoresis were carried out by standard methods (31, 4). DNA fragments were recovered from agarose gels with the QIAGEN gel extraction kit. Digoxigenin probe labeling and development of Southern blots were performed according to the manufacturer's instructions (Roche). Standard protocols were used for colony hybridization (31). Electrotransformation of freshly plated *Pseudomonas* cells was performed as previously reported (13).

Computer-assisted detection of IS*Ppu10* target. The genome sequence of *P. putida* KT2440 was screened in search for the 23-nucleotide sequence that corresponds to the IS*Ppu10* target, 5'-TTCGCGGGTAAACCCGCTCTAC-3', an internal part of the consensus for the REP sequences found in *P. putida* KT2440. Specific computer programs were developed to assist in the detection and analysis of IS*Ppu10* target sequences in the genome of KT2440 (3). This set of programs, which is available upon request, transforms a BLAST-generated output into an appropriate graphic format suitable to unequivocally distinguish if the target sequences are extragenic or intragenic. Open reading frames (ORFs) in the surroundings of the target were easily identified by means of their locus (PP) numbers as annotated by Nelson and coworkers (28), information obtained from the Institute for Genomic Research (TIGR), and a link was created to the BLAST results generated at the National Center for Biotechnology Information (NCBI).

Sequence analysis and comparisons. Gene sequence and physical organization data for *P. putida* were obtained from TIGR (www.tigr.org) and the NCBI. Sequences were also analyzed with the Omega program (2.0) and compared with the GenBank databases by using BLAST programs (2). IS general information and IS family classification were consulted at the IS Database (<http://www-is.biotoul.fr/is.html>).

Isolation of IS*Ppu10* from the KT2440 chromosome and construction of IS*Ppu10*-*km*. Several cosmids were isolated from a *P. putida* KT2440 gene library after selecting positive clones in a screen by colony hybridization with a digoxigenin-labeled IS*Ppu10* (mus9) PCR probe. The probe, 591 bp long, was amplified with the whole chromosome as the template with oligonucleotides mus9 (3+) (5'-ATGGCAATGTCCGCAATCC-3') and mus9 (4-) (5'-CGGAAGCC TCTGAACACG-3') at 60°C (annealing temperature) and 35 s of extension time. Two of the positive cosmids were selected for further work, pMIR72 and pMIR73. A 1.7-kb KpnI/BglII DNA fragment from pMIR72 containing IS*Ppu10* was cloned at KpnI/XbaI sites of plasmid pUC18 (39), which confers ampicillin resistance, resulting in plasmid pMIR101. A 1-kb SalI kanamycin resistance cassette from p345-Km3 (AF083408; 12) was then inserted into the unique XhoI site of pMIR101, which is located within the right end of IS*Ppu10*, 83 bp downstream from the stop codon of the transposase gene, generating IS*Ppu10*-*km* and giving rise to plasmid pMIR103. A 2.7-kb SalI/EcoRI fragment from pMIR103 containing IS*Ppu10*-*km* was cloned at the compatible sites of plasmid pUNØ18 (26) to generate pMIR112. This plasmid, pMIR112, which is suicidal in *Pseudomonas* and mobilizable when the *tra* functions of RK2 are supplied in *trans*, was further used to deliver IS*Ppu10*-*km* in tests of transposition.

Construction of plasmid-borne IS*Ppu10* target. A plasmid containing the IS*Ppu10* target sequence was constructed as follows. A 23-bp fragment was generated by hybridization of complementary oligonucleotides REP35E (5'-AA TTCGCGGGTAAACCCGCTCTAC-3') and REP35B (5'-GATCGTAGGAG CGGGTTTACCCGCG-3'; bold letters match protruding ends generated by digestion with EcoRI and BamHI, respectively). Annealing was performed with 4 µg of each primer in 20 µl of 10 mM Tris (pH 7.5)–15 mM NaCl–2.5 mM EDTA. After heating at 95°C for 10 min, the reaction mixture was slowly cooled down to 50°C and kept at this temperature for 5 h, which was followed by overnight incubation at 37°C. Thus, the fragment was cloned into the compatible sites of pBBR1-MCS5 (24), a plasmid that confers gentamicin resistance and is stably maintained in *Pseudomonas*, to generate pMIR111.

Target analysis and identification of flanking regions following in vitro transposition of IS*Ppu10*-*km*. Sequences at both ends of plasmid-borne IS*Ppu10*-*km*, in pMIR113, were determined with oligonucleotides ext5' antipmus9 (5'-CTAC

GGTCTGTACTGCGCGAAC-3') and ext3' dirmus9 (5'-CGTACCTCGAGGT TGCTAG-3'), which annealed at its left and right ends, respectively. Primers annealing within cloning vector sequences were also used as required. When characterizing the target and flanking sequences of chromosomally inserted IS*Ppu10*-*km*, an arbitrary PCR was used. A first round of amplification was done with chromosomal DNA as the template, with an arbitrary primer (ARB1 [5'-GGCACGCGTCTGACTAGTACNNNNNNNNNGATAT-3']) and an internal primer with annealing in the left end of IS*Ppu10* (5'-mus9ext [5'-GCTTGGGT GTTGAAAGGAGG-3']). The first round was as follows: 3 min at 95°C; six cycles of 1 min at 95°C, 1 min at 30°C, and 1 min at 72°C; 30 cycles of 30 s at 95°C, 30 s at 50°C, and 1 min at 72°C; and an extension period of 7 min at 72°C. A second round of amplification was done by using as the template 5 µl of the first-round reaction as follows: 3 min at 95°C; 30 cycles of 30 s at 95°C, 30 s at 60°C, and 1 min at 72°C; and 7 min at 72°C. Primers used for the second round were one corresponding to the conserved region of ARB1 (ARB2 [5'-GGCAC GCGTCTGACTAGTAC-3']) and a second, internal, primer closer to the border in the left end of IS*Ppu10* (5'-mus9int [5'-TTCTCTACGGTCTGTACTGCG-3']). Reaction mixtures were electrophoresed, and the most intense bands were isolated and sequenced. Sequencing was done on an ABI PRISM 310 automated sequencer with oligonucleotide 5'-mus9int as the primer. The 73-bp distance between this primer and the initiation of the IS target provided an internal control to ensure the legitimacy of the sequence obtained. To determine the sequence adjacent to the right end of IS*Ppu10*, oligonucleotides 3'-mus9ext (5'-GGGCAGTATCCAATGCTGG-3') and 3'-mus9 int (5'-GAAATTCTCTGGC ACGCTGAC-3') were used instead of 5'-mus9ext and 5'-mus9int, respectively.

RESULTS AND DISCUSSION

Identification and characterization of the repetitive element *mus-9* (IS*Ppu10*) in the *P. putida* KT2440 chromosome. Strain *mus-9* is a derivative of *P. putida* KT2440 obtained by random mutagenesis with mini-Tn5[Km1] (15). The gene where the minitransposon had been inserted (which was then designated *mus-9*) encodes a protein that was later annotated as a putative transposase (28), and the name IS*Ppu10* was assigned to the genetic element of which it is part. Seven identical copies of the gene were identified in the chromosome of KT2440 (28), corresponding to loci PP0526, PP1653, PP2134, PP3502, PP4599, PP5050, and PP5290 (the one mutated in strain *mus-9*), but no further characterization was done.

The existence of multiple 100% identical copies of IS*Ppu10* has allowed the precise definition of the borders of this genetic element and the performance of a detailed analysis of its structure. IS*Ppu10* is 1,339 bp long, with a lower G+C content (57%) than the average for the *P. putida* KT2440 chromosome (61.4%). It is not delimited by perfect inverted repeats, and a single ORF of 966 bp (including the stop codon TGA), corresponding to *mus-9*, appears to be present, thus leaving the left and right ends of IS*Ppu10* 221 and 152 bp in length, respectively. A putative ribosome binding site (AGGAG) is located 9 bp upstream of the initiation codon of *mus-9*. The 321-amino-acid protein encoded by this ORF is 26% identical to the pilin gene inverting protein PivNM-2 of *Neisseria meningitidis* (AAF42093), the IS621 transposase of *E. coli* (BAC76889), and the ISC1190 transposase of the archaeon *Sulfolobus solfataricus* (A90236), among others. In the Conserved Domain Database (25), a domain characteristic of pfam02371 (family of transposase 20) can be identified between amino acids 170 and 273 (Fig. 1). Partial similarity to a domain that is characteristic of pfam1548 (family of transposase 9, which includes several pilin gene inverting proteins) can also be found between residues 70 and 129, as well as the typical "signature" D-E (or D)-D-D sequence of the Piv/MooV family of transposases and site-specific recombinases (38). Although there is divergence in

FIG. 1. Characteristics of the transposase Mus-9 encoded in ISPpu10. The D-E-D-D motif is shaded black, amino acid residues present in all of the proteins in the alignment of transposases of the IS110/IS492 family and Piv proteins (Fig. 4 in reference 8) are indicated by asterisks, and other conserved amino acid residues are indicated by dots. A proline present in most proteins of the family is shown in parentheses. Gray shading indicates conserved domains within the transposase 9 family (pfam02371; light shading, black letters) and the transposase 20 family (pfam01548; dark shading, white letters).

the nucleotide sequences of these elements, the transposases encoded by them show partial homology to one another and, their most characteristic feature, to the *piv* gene invertase (Piv). The conserved aspartic and glutamic residues at certain positions appear to be directly involved in DNA recombination. These catalytic residues, as well as all other residues conserved in the Piv/MooV family of DNA recombinases (8), are present in the ISPpu10 transposase, except for a proline (Fig. 1). After aligning transposases encoded by IS110/IS492 members and proteins encoded by *piv* genes, Choi and coworkers (8) identified the motif DEDD, relating it to the one found in the catalytic center of the RuvC Holliday junction resolvase. In fact, a model for Piv-mediated inversion that includes resolution of a Holliday junction has been proposed (6).

Its structural features and the absence of perfect inverted repeats at both ends place ISPpu10 as a new member of the IS110/IS492 family of mobile genetic elements, being related to IS621 of *E. coli* (8).

The target sequence of ISPpu10 corresponds to a REP element. When analyzing the complete genome of KT2440, Nelson and coworkers (28) noted that ISPpu10 was flanked by sequences that were repeated throughout the chromosome. These sequences had been previously identified by Aranda-Olmedo et al. (3) as REP elements. These are highly conserved sequences 35 bp long and widespread throughout the chromosome of KT2440. By comparing all the copies of ISPpu10 (Fig. 2A), we could establish as a potential target for insertion a unique 23-bp sequence (TCGCGGGTAAACCCGCTCCTAC) that is identical to the proposed consensus for the central region of REP elements (3) and includes an internal palindrome (underlined). Insertion of ISPpu10 results in asymmetrical disruption of this sequence along with a 2-bp duplication (the CT pair in boldface), leaving 18 bp of the target sequence on the left side of ISPpu10 and 7 bp on the right side (Fig. 2B). Such specificity of insertion (REPs), although unusual, is shared with IS621 (like ISPpu10, a member of the IS110/IS492 family) from *E. coli* (8) and the IS3 family members IS1397 and ISKpn1 from *Enterobacteriaceae* (9, 41). Another *P. putida* KT2440 IS element, ISPpu9, is inserted into the same position in the REP as ISPpu10 but in the antiparallel strand and presents less-stringent target specificity (28).

In *Enterobacteriaceae*, REP sequences have been classified into three types (5), which often are organized in more complex mosaic elements called bacterial interspersed mosaic elements. The highest stringency for the type of REP was exhibited by ISKpn1 from *Klebsiella pneumoniae*, followed by IS621, being the most permissive IS1397 from *E. coli* (8, 40), which

can transpose to any of the three REP types. In *P. putida*, only eight bacterial interspersed mosaic elements were found and most of the REP sequences appear as single elements or pairs, the most common organization of which is convergent or divergent rather than directly repeated (3). ISPpu10 exhibits highly stringent target sequence specificity, but insertion seems to be independent of further REP organization, occurring either at single divergent or convergent pairs or clusters of REPs (not shown).

The possibility has been discussed that this strategy of insertion in REPs, extragenic as they are for the most part, represents a propagation strategy that avoids harm to the host cell, as the insertion events would occur outside essential genes.

Genomic analysis for potential ISPpu10 targets. A search of the chromosome of KT2440 identified 205 potential targets for ISPpu10. According to TIGR annotation (November 2004, as recorded by the NCBI), 18 are intragenic and located in 15 different ORFs (Table 1). These ORFs were analyzed in detail to examine a putative effect of the presence of the ISPpu10 target upon the deduced amino acid sequence. Protein sequences were compared with the databases by using the BLASTP program. The fact that 10 targets were contained in seven putative proteins, 6 of which are shorter than 75 residues (Table 1) and none of which exhibit similarity to peptides from other organisms, strongly suggests misannotation of these proteins. This comes to support the proposal by Tobes and Pareja (36) that the presence of REPs might be used for genome reannotation. Based on ISPpu10 target position and homology to other proteins in the databases, reannotation of PP0525 and PP1761 is also suggested (Table 1). These two polypeptides showed extensive similarity to proteins in the databases. However, they were longer than their homologs, with tails of residues in their amino termini of 26 and 167 residues, respectively, which exhibited no similarities to other polypeptides in the databases. In PP0525, a putative TonB-dependent receptor of the B12 family, an ISPpu10 target was located starting at position 22 downstream from the annotated start codon. However, a putative in-frame initiation codon is located 85 bp downstream from the annotated one, 6 bp from a potential Shine-Dalgarno consensus sequence. Thus, we propose this second ATG as the actual start codon of PP0525. Regarding PP1761, a putative GGDEF sensor protein, a search for alternative in-frame initiation points identified an ATG codon, preceded by a Shine-Dalgarno consensus, located 465 bp downstream from the annotated start codon. In four cases, PP0101, PP1518, PP3753, and PP4398, the stops codons were part of the ISPpu10 target and no size reduction was observed

A

Coordinates	Left end (221 bp).....	966 bp	Right end (152 bp).....	Coordinates
610869	GCCGCTACCGTATTCGCGGGTAAACCCGCTTGTATGGTAG...	<i>mus-9</i>	...GCCATAGAATCTCCTACAGTGGGCACACG	612231
1847043	TGTGCCGGCCTCTTCGCGGGTAAACCCGCTTGTATGGTAG...	<i>mus-9</i>	...GCCATAGAATCTCCTACAGGCTTGCGAAT	1848405
2435221	CGCGGTGCCTGCTTCGCGGGTAAACCCGCTTGTATGGTAG...	<i>mus-9</i>	...GCCATAGAATCTCCTACGGGGGCATCCAG	2436583
3971761	AGGCCCGGCTCTTCGCGGGTAAACCCGCTTGTATGGTAG...	<i>mus-9</i>	...GCCATAGAATCTCCTACAGGGGCTTGTGG	3973123
5218866	TGTTCTGGCCTCTTCGCGGGTAAACCCGCTTGTATGGTAG...	<i>mus-9</i>	...GCCATAGAATCTCCTACATCGGCAACAGG	5220228
5756769	GCCACCACCGTATTCGCGGGTAAACCCGCTTGTATGGTAG...	<i>mus-9</i>	...GCCATAGAATCTCCTACCCCAATCCTGTG	5758131
6032836 (rc)	AGTACGGGCCCTTTCGCGGGTAAACCCGCTTGTATGGTAG...	<i>mus-9</i>	...GCCATAGAATCTCCTACAGGGGTAGCGTC	6032790
6039204 (rc)	TGATCTGGCCCTTTCGCGGGTAAACCCGCTTGTATGGTAG...	<i>mus-9</i>	...GCCATAGAATCTCCTACATGGGGATTTC	6037842

B

REP consensus (ISPpu10 target):

5'-cCGGCCTcTTCGCGGGTAAACCCGCTCCTACAGGg-3'
 3'-gGCCGgagAAGCGCCATTGGGCGAGGATGTCc-5'

TTCGCGGGTAAACCCGCT **ISPpu10** CTCCTAC

C

FIG. 2. Detection of multiple copies of ISPpu10 elements in the chromosome of *P. putida* KT2440. (A) Flanking sequences of ISPpu10, coordinates of the first and last bases shown as annotated for *P. putida* KT2440 by TIGR. Black-shaded white letters are REPs, and the CT duplication is in bold and underlined. The left and right ends of ISPpu10 are shaded in pale gray. The extra copy of ISPpu10 in the Granada variant is shown in dark gray shading; rc indicates that the sequence shown is in the reverse complementary strand. This region containing the extra copy was PCR amplified and sequenced with oligonucleotides radC(-) (5'-GCATTGACGCTGTCGTTGAAG-3') and upmus9(+) (5'-GCAGACA CTGATCCAGCAGCAG-3'). Numbers are coordinates of the Mus-9 transposase, the transposase encoded by ISPpu10, as annotated by Nelson and coworkers (28). (B) ISPpu10 target sequence (shaded black), REP consensus, and probable secondary structure adopted by the palindrome. (C) Pattern variability of ISPpu10 elements in *P. putida* KT2440. Ten-microgram samples of DNA from diverse subtypes of KT2440 were digested with PstI, which does not cut within ISPpu10. Hybridization was performed with an internal digoxigenin-labeled ISPpu10 PCR probe as indicated in Materials and Methods. Lane 1, Granada variant of KT2440 (ancestor of mus-9); lane 2, mutant mus-9; lane 3, another variant of KT2440 from our laboratory; lane 4, a rifampin-resistant derivative from Denmark. Sizes of molecular weight marker (λ DNA digested with HindIII) bands are indicated in kilobases on the left. Arrowheads indicate bands in the Granada variant and those appearing differentially in distinct variants; stars indicate missing bands.

by comparison with their homologs. The most interesting coding effect was related to the REP located in PP1718, which has similarities to a family of bacterial signaling proteins characterized by the presence of an EAL motif, which has been shown to be involved in turnover (degradation) of the cyclic nucleotide c-di-GMP (34). In PP1718, a truncation longer than 200 residues, which involved a C-terminal GGDEF domain, was observed in association with the intragenic ISPpu10 target. Such a deletion could be the consequence of homologous recombination between two directly repeated copies of ISPpu10. In other proteins of the family, the GGDEF domain is related to the production of c-di-GMP (34). Transitions from sessility

to motility mediated by c-GMP levels have been observed in *P. aeruginosa* and enteric bacteria (34). It could be that losing the GGDEF domain for the production of c-di-GMP was a beneficial result and consequently was selected and fixed in the population of KT2440.

Variability in the number and distribution of ISPpu10 elements in different subtypes of KT2440. During the characterization of the mus-9 mutant, Southern analysis showed that the KT2440 strain being used in our laboratory, and its derivative mus-9, had eight copies of the gene instead of the seven copies found in the published genome sequence of KT2440 (Fig. 2). Furthermore, in our laboratory stock collection there were

TABLE 1. ORFs in *P. putida* KT2440 genome containing REPs

Locus no.	PP coordinates ^a	REP coordinates ^a	Hit ^b	Reannotation suggested
PP1172	1346975–1347190	1347209–1347187 ^c 1347116–1347138 1347057–1347035	NO	No gene
PP1704	1903494–1903619	1903559–1903537	NO	No gene
PP1848	2073464–2073577	2073524–2073502	NO	No gene
PP2850	3252514–3252627	3252617–3252639 ^d	NO	No gene
PP2878	3279248–3279430	3279386–3279408	NO	No gene
PP3479	3943877–3944410	3944287–3944265	NO ^c	
PP4238	4811756–4812079	4811996–4812018 4811937–4811915	NO	No gene
PP5049	5756580–5756687	5756649–5756671	NO	No gene
PP0101	106133–107650	107669–107647 ^c	Sulfate transporter (putative)	
PP0525	608918–610843	610822–610800	TonB-dependent receptor	608918–610759
PP1518	1724478–1725164	1724459–1724481 ^c	Conserved hypothetical protein	
PP1718	1917139–1918278	1917221–1917243	Signaling protein	
PP1761	1963390–1966311	1963699–1963721	Sensory box protein	1963855–1966311
PP3753	4282139–4283227	4282149–4282127 ^d	AraC/XylS family regulator	
PP4398	4988422–4989756	4988403–4988425 ^c	Permease of major facilitator superfamily	

^a Coordinates have been determined referring to *P. putida* KT2440 sequenced by TIGR.

^b Result of BLAST analysis with proteins of other organisms.

^c In *P. fluorescens* and other related bacteria, a hypothetical protein with no conserved domain is annotated in the antiparallel strand.

^d A stop codon (TAA) is in the REP sequence.

^e A stop codon (antiparallel CTA) is in the REP sequence.

KT2440 variants, obtained from various sources, with either seven copies or an eighth copy of the gene in different locations, as shown by restriction fragment length polymorphism analysis with the ISPpu10 element as the probe (Fig. 2C). Such polymorphism indicates that ISPpu10 is, in fact, a mobile genetic element responsible for genotypic variation in *P. putida*.

Two cosmids were isolated from a KT2440 gene library containing the wild-type allele of ISPpu10 which had been interrupted in strain mus-9 (PP5290). The restriction map of this region is shown in Fig. 3A. One of the cosmids, pMIR73, presented the expected 6.6-kb PstI band containing ISPpu10 (Fig. 3B). However, in the other cosmid, pMIR72, an extra 2.7-kb PstI band showed a hybridization signal when ISPpu10 was used as the probe, indicating that an extra copy was located relatively close to the copy mutated in mus-9 (PP5290), both copies being within a single 8.1-kb KpnI restriction fragment. This extra copy of ISPpu10 is located between genes PP5283 and PP5284.

The comparison between the sequenced variant of KT2440 and the Granada variant of KT2440, in the chromosomal region where the extra copy of ISPpu10 is present in the latter, provided further evidence of the target sequence for insertion of ISPpu10. In this location, the KT2440 sequence contains a REP element with an internal 23-bp sequence corresponding

to the proposed target for ISPpu10. In the Granada genotype, this sequence appeared asymmetrically interrupted by the extra copy of the transposon, along with the predicted 2-bp CT duplication at the junction region of ISPpu10 with the target sequence (Fig. 2A).

Diverse *P. putida* strains were analyzed by Southern blotting for the presence of ISPpu10. *P. putida* JLR11 (16) showed a total of seven copies, giving the same hybridization pattern as a *P. putida* KT2442 variant from Denmark (11). However, *P. putida* strains DOT-T1E (30), F1 (18), SMO116, MTB5, and MTB6 (22) did not show any element of ISPpu10 (not shown).

Experimental evidence of ISPpu10 transposition activity to its target in a plasmid and in the chromosome. The genotypic variability observed in KT2440 subtypes indicated that ISPpu10 has, or has in the recent past had, transposition activity. In order to obtain experimental evidence that ISPpu10 is in fact an active transposable element, two genetic tools were developed, as described in Material and Methods, which allowed us to select for transposition events, i.e., ISPpu10-*km*, a kanamycin-resistant derivative of ISPpu10 that was introduced in a mobilizable suicide plasmid into *Pseudomonas*, pMIR112, and a plasmid-borne target in pMIR111. Plasmid pMIR111 was introduced by electrotransformation into *P. putida* DOT-T1E (30), a strain closely related to KT2440 but where the presence

FIG. 3. Region containing the extra copy of ISPpu10 in the Granada genotype of KT2440. (A) Map of cosmid pMIR72. (B) Hybridization pattern of cosmids pMIR72 (lanes 1 and 3) and pMIR73 (lanes 2 and 4) against a *mus-9* probe. Southern blot assay of the cosmids digested with PstI (lanes 1 and 2) or with EcoRI and BglII (lanes 3 and 4). A digoxigenin-labeled PCR product amplified with primers *mus9* (3+) and *mus9* (4-) was used as the probe. Molecular weight markers were as indicated in the legend to Fig. 2C.

of an ISPpu10 homolog was ruled out by hybridization and PCR (not shown). DOT-T1E(pMIR111) was then used as the recipient in a triparental conjugation with *E. coli* DH5α harboring pMIR112 (which is unable to replicate in *Pseudomonas*) as a donor and HB101(RK600) as a helper strain. Km^r Gm^r exconjugants of DOT-T1E(pMIR111) were selected with a frequency of 10⁻⁶ per recipient by using 10 mM citrate-supplemented M9 minimal medium to counterselect the *E. coli* donor and helper strains. Several clones were analyzed by Southern blotting to confirm that ISPpu10-*km* had been acquired. As expected, transconjugants carried a mixture of pMIR111 and a derivative of this plasmid carrying ISPpu10-*km*, which was named pMIR113 (Fig. 4A). Plasmids from one of these transconjugants containing pMIR113 were isolated and used to transform DH5α cells in order to select for pMIR113, thus avoiding the mixture with pMIR111. The insertion site within plasmid pMIR113 was identified by sequencing with universal and reverse primers from sequences in the vector. The predicted insertion of ISPpu10 in the REP core sequence, with the subsequent CT base pair duplication, was

thus confirmed. The same CT duplication was always associated with ISPpu10 insertion. Although a similar observation had been made for IS621 and two other members of the IS110/IS492 family (8), active transposition into the target could not be demonstrated in those cases.

Interestingly, genetic diversity was observed among transconjugants. In some cases, the hybridization band was not of the size and intensity expected for a plasmid-inserted copy of ISPpu10-*km*, whereas in others more than one band was detected (Fig. 4B). These data suggested that ISPpu10-*km* had not only been inserted into the plasmid-borne target sequence but could also integrate into the chromosome of DOT-T1E, supporting the predicted presence of REPs in this strain, deduced indirectly from REP PCR DNA fingerprinting (3).

Transposition of ISPpu10-*km* into the chromosome of DOT-T1E was confirmed by introducing plasmid pMIR112 into this strain and further selecting for kanamycin resistance as described above. Kanamycin-resistant clones were recovered with a frequency of 4 × 10⁻⁷ exconjugants per recipient. The integration of ISPpu10-*km* into the chromosome of DOT-T1E was

FIG. 4. Insertion of ISPpu10 into a pMIR111-borne target. (A) After transferring pMIR112-borne ISPpu10-*km* from *E. coli* DH5 α to DOT-T1E(pMIR111), total DNAs from three independent kanamycin-resistant exconjugants were digested with PstI (lanes 1 to 3). Plasmids pMIR111 and pMIR113 present a unique PstI restriction site. (B) Hybridization patterns exhibited by the exconjugants against the same probe as in Fig. 3. The light hybridization band in lane 1 corresponds to ISPpu10-*km* inserted into the chromosome; the exconjugant shown in lane 2 exhibits a pMIR113-borne copy (larger band) and another in the chromosome (smaller); a unique pMIR113-borne copy is observed in lane 3. Molecular weight markers were as indicated in the legend to Fig. 2C.

confirmed by PCR amplification with oligonucleotides mus9 (3+) and mus9 (4-). Diversity of DOT-T1E derivatives with ISPpu10-*km* integrated in the chromosome could be observed (not shown). Sequencing of the region flanking ISPpu10 in several clones showed that insertion had taken place in REP sequences at different chromosomal locations, all of them intergenic, and interestingly, one corresponded to the same location as the copy mutated in the KT2440 mus-9 derivative (not shown). Confirmation of the presence in *P. putida* DOT-T1E of specific REP sequences containing the ISPpu10 target was also possible at the position occupied by the extra copy in our KT2440 variant (not shown).

DNA rearrangements observed in relation to ISPpu10. The mobile character of ISPpu10, its similarity to recombinases, and the existence of two copies in the same orientation and relatively close in the chromosome of the Granada genotype of KT2440 opened the possibility that this region suffered rearrangements or was unstable. In fact, although cosmid pMIR72 (Fig. 3A) harboring both copies of ISPpu10 was stably maintained in *E. coli*, deletions have been observed after cloning fragments containing two copies of the IS in multicopy vectors. Distinct deletions were also observed in fragments containing a single copy of ISPpu10 (our unpublished results). Different findings have previously suggested a role for DNA rearrangements in plant-associated *Pseudomonas* populations (reviewed in reference 14). Phenotypic variability has been observed in *P. fluorescens* in the rhizosphere of alfalfa plants (32), and a site-specific recombinase was found to be relevant for competitive colonization of tomato root tips by *P. fluorescens* (10) and *Pseudomonas chlororaphis* PCL1391 (7). We have also observed rearrangements mediated by ISPpu10 to take place in *P. putida* during colonization of the corn rhizosphere (not shown), and although a detailed characterization of these rearrangements has yet to be done, the possible role of ISPpu10

in the adaptation and evolution of plant-associated *P. putida* populations is an exciting avenue for future research.

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