

## A Third Transposable Element, *ISPpu12*, from the Toluene-Xylene Catabolic Plasmid pWW0 of *Pseudomonas putida* mt-2

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A 3,372-bp insertion sequence, *ISPpu12*, has been identified on the archetypal toluene-xylene TOL catabolic plasmid pWW0 from *Pseudomonas putida* mt-2. The insertion sequence element is located on the plasmid between bases 84397 and 87768 in a region which also contains the termini and transposase genes of the catabolic transposons Tn4651 and Tn4653 (A. Greated, L. Lambertson, P. A. Williams, and C. M. Thomas, *Environ. Microbiol.*, in press). *ISPpu12* has terminal inverted repeats of 24 bp with three mismatches and contains four open reading frames, a *tnpA* homologue and three open reading frames (*lspA*, *orf1*, and *orf2*) of undetermined function. After insertion in vitro of a Km<sup>r</sup> cassette into *ISPpu12* either in the intergenic region between *orf1* and *orf2* or directly into the *orf1* gene and ligation into a suicide vector, the modified *ISPpu12*-Km transposes at high frequency, often in multiple copies, into the chromosome of a *P. putida* recipient. Inactivation of *lspA*, *orf1*, and *orf2* by introducing a 7-bp deletion into the 5' region of each gene had no major effect upon transposition, but a similar mutation of *tnpA* completely eliminated transposition. Analysis of the literature and of strains derived from the chlorobenzoate-degrading *Pseudomonas* sp. strain B13 suggests that the promiscuity of this element has played an important role in the history of plasmid pWW0. Database comparisons and the accompanying paper (A. J. Weightman, A. W. Topping, K. E. Hill, L. L. Lee, K. Sakai, J. H. Slater, and A. W. Thomas, *J. Bacteriol.* 184:6581–6591, 2002) show that *ISPpu12* is a transposable element also found in other bacteria.

The 116,580-bp IncP9 plasmid pWW0 from *Pseudomonas putida* mt-2 (8) is an archetype of the large group of catabolic plasmids found widely in many genera of gram-negative soil bacteria. It has been much studied, mainly because of the four *xyl* regulons which encode the inducible catabolism of toluene and some substituted toluenes to central metabolites (2, 33). Studies on the two operons of structural genes and the regulation of their expression by the gene products of *xylS* and *xylR* have made this arguably one of the best understood long catabolic pathways for substrates of nonbiological origin. In addition to its catabolic function, the plasmid has a number of other interesting structural features. The complete *xyl* genes are located within a 39-kb span between two directly repeated copies of a 1,275-bp insertion sequence, *IS1246* (23). The only reported phenotype resulting from this arrangement is that the entire catabolic region can be deleted from the plasmid by homologous recombination between the direct repeats. The result is a smaller, but still conjugative, 78-kb plasmid, pWW0-8, which is phenotypically cryptic (3, 7) and carries only a single copy of *IS1246*. There are two large nested transposons on pWW0. Tn4651 is 56 kb and encompasses the 39-kb catabolic region (27), and Tn4653 is 70 kb and spans Tn4651 (28). Both are class II (Tn3-like) transposons which share common *tnpT*, *tnpS*, and *res* genes but have individual *tnpR* and *tnpA* genes and are bounded by terminal inverted repeats of 46 and 38 bp, respectively (29). Although both carry the 39-kb catabolic region and both confer the ability to grow on the TOL

substrates when they transpose, their transposition genes are not within the catabolic region and both transpose equally well when the 39-kb catabolic region has been deleted, when they are referred to as Tn4652 and Tn4654, respectively (27, 28).

In a number of early studies (15, 17, 22) pWW0 was found to carry insertions of 3 to 3.5 kb in various locations, usually within the catabolic regions, where their presence has been associated with a change in the catabolic phenotype. The only clue as to the origin of an insertion of this size was from Southern blots of several such plasmids, which suggested that they hybridized with a region of pWW0 well separated from the catabolic region (17). Analysis of the recently completed nucleotide sequence of pWW0 (accession number AJ344068) has suggested that the plasmid has had a complex evolution and that multiple transposition and recombination events have made a major contribution to its present structure (8).

The availability of the complete sequence has facilitated our reexamination of one ~3-kb insertion in a plasmid derived from pWW0 (15) and its comparison with the wild-type pWW0 sequence. As a result, we report that pWW0 carries a functional 3,372-bp insertion sequence, *ISPpu12*, which is located between bases 84397 and 87768 in the annotated sequence.

### MATERIALS AND METHODS

**Bacterial plasmids and strains.** The bacterial plasmids used and constructed in this study are listed in Table 1. *Escherichia coli* DH5 $\alpha$  [ $\phi$ 80d*lacZ*AM15 *recA1 endA1 gyrA96 thi-1 hsdR17*(r<sub>K</sub><sup>-</sup> m<sub>K</sub><sup>+</sup>) *supE44 relA1 deoR*  $\Delta$ (*lacZYA-argF*)U169] (Invitrogen) was used routinely as a host in cloning experiments.

**Media and bacterial culture.** *Pseudomonas* strains were grown on minimal medium supplemented with either 10 mM succinate, 5 mM 3-chlorobenzoate, or 4-chlorobenzoate as required. Liquid Luria-Bertani medium or Sensitivity Test agar (LabM, Bury, United Kingdom) was used for the cultivation of *E. coli*

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TABLE 1. Strains and plasmids used in this study

Bacterium or plasmid	Description	Reference(s) and/or source
<b>Bacteria</b>		
<i>P. putida</i> mt-2 (ATCC 33015)	Wild-type strain carrying TOL plasmid pWW0	33, 34
<i>P. putida</i> PaW82	Plasmid-free derivative of <i>P. putida</i> mt-2	3
<i>P. putida</i> PaW340	Str <sup>r</sup> Trp <sup>-</sup> derivative of PaW85	14
<i>Pseudomonas</i> sp. strain WR211	Primary transconjugant of <i>Pseudomonas</i> sp. strain B13 derived from mating pWW0 from <i>P. putida</i> mt-2; carries plasmid pWW0-1211	15, 25
<i>Pseudomonas</i> sp. strain WR216	Carries plasmid pWW0-1216; derived from WR211 by selection for acquired ability to grow on 4-chlorobenzoate; strain appears no longer to exist.	15, 25
<i>Pseudomonas</i> sp. strain WR216-2	Carries plasmid pWW0-1216/2; variant of WR216 obtained from old slope	15, 25
<i>Pseudomonas</i> sp. strain WR216-3	Carries plasmid pWW0-1216/3; new variant of WR216 selected from WR211 for acquired ability to grow on 4-chlorobenzoate	This study
<i>P. putida</i> PRS2000	Wild type (PRS1, A.3.12)	L. N. Ornston
<i>P. putida</i> PRS2000TK1 to -20	Series of Km <sup>r</sup> transconjugants with IS <i>Ppu12</i> -Km integrated into chromosomes	This study
<i>E. coli</i> CC118λpir	Maintenance strain for pGP704	9
<i>E. coli</i> S17-1λpir	Donor strain for pGP704	9
<i>E. coli</i> DH5α		Invitrogen
<b>Plasmids</b>		
pUC18	Cloning vector; Ap <sup>r</sup>	35
pUC18K	pUC18 with <i>KpnI</i> site in MCS removed	R. M. Jones
pUC4K	Ap <sup>r</sup> Km <sup>r</sup> plasmid carrying Km <sup>r</sup> cassette	30; Promega
pGP704	Suicide vector; Ap <sup>r</sup>	18
p34SKm	Plasmid carrying Km <sup>r</sup> cassette	5
pGEMTeasy	Vector for cloning PCR products; Ap <sup>r</sup>	Promega
pXQ1	5.4-kb <i>XhoI</i> -Q fragment of pWW0-1216-2 cloned into <i>SalI</i> site of pUC18	This study
pXQ2	4.58-kb <i>SalI</i> fragment from pXQ1 cloned into <i>SalI</i> site of pUC18	This study
pXQ3	4.58-kb <i>SalI</i> fragment from pXQ2 cloned into <i>SalI</i> site of pUC18K	This study
pXQ3Km1	Km <sup>r</sup> cassette from p34SKm inserted into <i>KpnI</i> site of pXQ3 in same orientation as <i>tnpA</i>	This study
pXQ3Km2	Km <sup>r</sup> cassette from p34SKm inserted into <i>KpnI</i> site of pXQ3 in opposite orientation to <i>tnpA</i>	This study
pGEMM3Km1	Km <sup>r</sup> cassette from pUC4K inserted into <i>BamHI</i> site in <i>orf1</i> of pGEMM3 in same orientation as <i>orf1</i>	This study
pGEMM3Km2	Km <sup>r</sup> cassette from pUC4K inserted into <i>BamHI</i> site in <i>orf1</i> of pGEMM3 in opposite orientation to <i>orf1</i>	This study
pXQ3Km3	<i>KpnI</i> - <i>MfeI</i> fragment of pGEMM3Km1 replacing <i>KpnI</i> - <i>MfeI</i> fragment of pXQ3	This study
pXQ3Km4	<i>KpnI</i> - <i>MfeI</i> fragment of pGEMM3Km2 replacing <i>KpnI</i> - <i>MfeI</i> fragment of pXQ3	This study
pGPTKm1	<i>SalI</i> fragment from pXQ3Km1 cloned into pGP704; suicide vector for delivery of IS <i>Ppu12</i> -Km1	This study
pGPTKm2	<i>SalI</i> fragment from pXQ3Km2 cloned into pGP704; suicide vector for delivery of IS <i>Ppu12</i> -Km2	This study
pGPTKm3	<i>XbaI</i> - <i>SphI</i> fragment from pXQKm3 cloned into pGP704; suicide vector for delivery of IS <i>Ppu12</i> -Km3	This study
pGPTKm4	<i>XbaI</i> - <i>SphI</i> fragment from pXQKm4 cloned into pGP704; suicide vector for delivery of IS <i>Ppu12</i> -Km4	This study
pGEMM1	2.0-kb overlap extension mutagenesis PCR product containing 7-bp deletion and novel <i>BamHI</i> site in <i>tnpA</i> , cloned into pGEMTeasy	This study
pGPM1	2.0-kb <i>XbaI</i> - <i>MfeI</i> fragment from pGEMM1 replacing wild-type fragment in pGPTKm2	This study
pGEMM2	1.6-kb overlap extension mutagenesis PCR product containing 7-bp deletion and novel <i>BamHI</i> site in <i>lspA</i> , cloned into pGEMTeasy	This study
pGPM2	1.6-kb <i>MfeI</i> - <i>XhoI</i> fragment from pGEMM2 replacing wild-type fragment in pGPTKm2	This study
pGEMM3	1.6-kb overlap extension mutagenesis PCR product containing 7-bp deletion and novel <i>BamHI</i> site in <i>orf1</i> , cloned into pGEMTeasy	This study
pGPM3	1.6-kb <i>MfeI</i> - <i>XhoI</i> fragment from pGEMM3 replacing wild-type fragment in pGPTKm2	This study
pGEMM4	1.3-kb overlap extension mutagenesis PCR product containing 7-bp deletion and novel <i>BamHI</i> site in <i>orf2</i> , cloned into pGEMTeasy	This study
pGPM4	1.3-kb <i>PstI</i> - <i>XhoI</i> fragment from pGEMM4 replacing wild-type fragment in pGPTKm1	This study
pTK16S1	~6-kb <i>SalI</i> fragment from genomic DNA of <i>P. putida</i> PRS2000TK16 cloned into pUC18	This study
pTK16S2	~6-kb <i>SalI</i> fragment from genomic DNA of <i>P. putida</i> PRS2000TK16 cloned into pUC18	This study

TABLE 2. Oligonucleotide primers used in this study

Purpose	Direction <sup>a</sup>	5'→3' sequence <sup>b</sup>	Equivalent position in pWW0 sequence (accession number AJ344068)
<i>orf1</i> probe for blotting	Reverse	GCGCGCCGGCGGTGATGAC	85778–85761
	Forward	AGGATGCGGCAGCGACGAATAAAG	84725–84748
External primer for OEM <sup>c</sup> of <i>tnpA</i>	Reverse	GATTACGAATTCGAGCTCGGTACC <sup>d</sup>	Derived from MCS of pGPTKm2 844157–86328
	Forward	TGATATTGCAATTGTCCTCGGTGCT	
Internal mutagenesis of <i>tnpA</i>	Reverse	GCCCCAGGA <sup>t</sup> Cc-Δ7-GGCGTTGAAGATGTGCGG	86844–86808
	Forward	TCAACGCC-Δ7-gGaTCCTGGGGCGCTGGCACCG	86818–86854
External primer for OEM of <i>IspA</i> and <i>orf1</i>	Reverse	CCGAGGACAATTGCAATATCAGCCAGG	86323–86297
	Forward	CGGCCTCGAGCAAGACGTTTCCCGT	Derived from Km <sup>r</sup> cassette
Internal mutagenesis of <i>IspA</i>	Reverse	CGATATGGAT <sup>t</sup> cc-Δ7-TACGGCGAGAGCTTTTGGCA	85938–85898
	Forward	CTCTCGCCGTA-Δ7-ggaTCCATATCGGGCCTGCTG	85947–85908
Internal mutagenesis of <i>orf1</i>	Reverse	GCATAACA-Δ7-ggaTCCCTGGCGCCTCGGAGGAGG	85093–85054
	Forward	GCGCCAGGG <sup>t</sup> Atcc-Δ7-TGTTTATGCCGTGCCGAAG	85065–85103
External primer for OEM of <i>orf2</i>	Reverse	GCGGGCCTCGAGCAAAGACGTTTCCCGTTGA	Derived from Km <sup>r</sup> cassette
	Forward	GCATGCCTGCAGGTGCACCAGTCCCT	Derived from MCS of pGPTKm1
Internal mutagenesis of <i>orf2</i>	Reverse	TCGGCA-Δ7-GGaTcCCGTGTCTATACCGAGAAACAC	84802–84763
	Forward	GGTATAGACACGG <sup>t</sup> AtCC-Δ7-TGCCGATCAGGCGGCG	84772–84812
Sequencing out from IRR	Reverse	ACGATGACCGGGAAGTTGAGG	84554–84534
Sequencing out from IRL	Forward	GCCGGACTTCGCAGAGGA	87659–87676

<sup>a</sup> Forward primers operate from left to right relative to Fig. 1.

<sup>b</sup> For the external primers for overlap extension mutagenesis, the relevant restriction sites (see Fig. 3) are in boldface. For the internal mutagenesis primers, the deletion of 7 bp is indicated by -Δ7- and the constructed *Bam*HI site is underlined, with the bases which are changed from the wild-type sequence to form it indicated in lowercase letters. Forward primers function from left to right relative to Fig. 1.

<sup>c</sup> OEM, overlap extension mutagenesis.

<sup>d</sup> The *Xba*I site used for the overlap extension mutagenesis of *tnpA* (see Fig. 3) is within the PCR fragment and not on the primer.

strains. Where appropriate, ampicillin (100 μg/ml) or kanamycin (15 μg/ml) was added.

**DNA extraction and manipulations.** Plasmid DNA was prepared from *Pseudomonas* by the method of Wheatcroft and Williams (32). Plasmids in *E. coli* were prepared by using the Concert rapid plasmid miniprep system (Life Technologies). Genomic DNA was prepared from *Pseudomonas* by using the QIAamp tissue kit (Qiagen). Restriction endonuclease digestions and ligations with T4 ligase were done in accordance with the instructions of the manufacturer (Promega). DNA fragments were recovered from agarose gels by using the QIAquick gel extraction kit (Qiagen). Southern blotting was performed as described by Sambrook et al. (26). Hybridization of blots used enhanced chemiluminescence direct labeling (Amersham). All other DNA manipulations were by standard procedures. PCR-generated fragments were routinely cloned directly into pGEMTeasy vector (Promega).

**DNA sequencing and analysis.** Nucleotide sequences were determined by MWG-Biotech Ltd. (Ebersburg, Germany). PCR primers (Table 2) were designed with the aid of the Lasergene software (DNASTAR, Madison, Wis.). Searches of databases were carried out with the BLAST programs (1).

**Construction of Km<sup>r</sup> derivatives of IS*Ppu12*.** (i) **pGPTKm1 and pGPTKm2.** The *Xho*I fragment of pWW0-1216 containing IS*Ppu12* was cloned into the *Sal*I site of pUC18 to form plasmid pXQ1. Using internal *Sal*I sites, a smaller internal 4.6-kb fragment was excised and recloned into the *Sal*I site of pUC18, forming pXQ2. From this, it was recloned into pUC18K, a derivative of pUC18 with the *Kpn*I site deleted from the multiple cloning site (MCS) to form pXQ3. Into its single *Kpn*I site, between *orf3* and *orf4*, a cassette carrying the kanamycin resistance cassette from p34SKm (5) was inserted. Two plasmids were formed, with the Km<sup>r</sup> gene oriented in the same direction as *tnpA* in pXQ3Km1 and in the opposite direction in pXQ3Km2 (Fig. 1). The *Sal*I insert of each of these

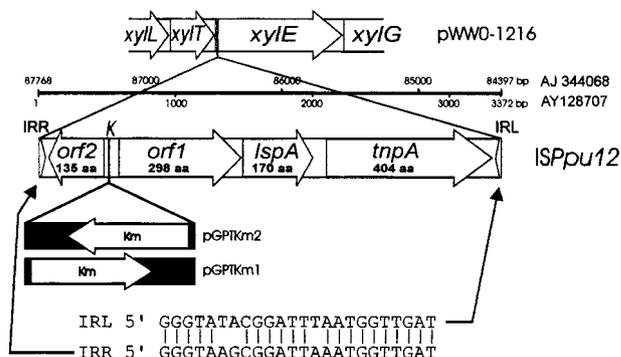


FIG. 1. Genetic map of IS*Ppu12*. The location and orientation of IS*Ppu12* relative to the *xyl* genes of the *meta* pathway operon on plasmid pWW0-1216, from which it was first isolated, are shown at the top. The bases are numbered according to GenBank accession numbers AJ344068 (complete pWW0 sequence) and AY128707 (IS*Ppu12*). The sequences of the two terminal inverted repeats are given below the map. The location of the Km<sup>r</sup> cassette in the unique *Kpn*I site between *orf1* and *orf2* to form IS*Ppu12*-Km on plasmids pGPTKm1 and pGPTKm2 is indicated. aa, amino acids.

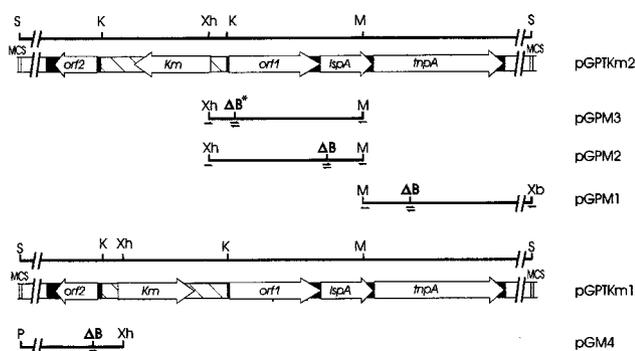


FIG. 2. Maps of deletion mutants of plasmids carrying the  $Km^r$  derivatives of IS*Ppu12*. Overlap extension PCR was used to generate fragments (shown as thin lines) with terminal restriction sites corresponding to unique sites within the two parental plasmids pGPTKm2 and pGPTKm1; within each fragment a 7-bp deletion adjacent to a unique *Bam*HI site was engineered in the 5' end of one of the ORFs to inactivate it (see Table 2). The PCR-generated fragment was spliced into pGP704 on the two terminal restriction sites to replace the wild-type DNA, forming plasmids pGPM1 to -4. The inserts were excised with the same restriction enzymes and inserted into one of two IS*Ppu12*-*Km* constructs, pGPTKm2 or pGPTKm1, replacing the equivalent wild-type DNA. Black area, IS*Ppu12* DNA; hatched area,  $Km^r$  cassette inserted into the IS*Ppu12* *Kpn*I site; shaded areas, MCS of the vector plasmid pGP704. The locations of the four primers used for each overlap extension PCR (see Table 2) are indicated by the half arrows, and  $\Delta B$  shows the position of the constructed *Bam*HI site deletion. The  $\Delta B$  with an asterisk on pGPM3 is the site at which the  $Km^r$  cassette is inserted in pGPTKm3 and pGPTKm4. Abbreviations for restriction sites: S, *Sal*I; K, *Kpn*I; M, *Mfe*I; Xb, *Xba*I; Xh, *Xho*I.

plasmids were recloned into the suicide vector pGP704 (18), forming pGPTKm1 and pGPTKm2, respectively. These were transformed into *E. coli* strains CC118*λpir* (for maintenance) and S17-1*λpir* (for mobilization).

(ii) **pGPTKm3 and pGPTKm4.** In order to insert a  $Km^r$  gene within *orf1*, the starting plasmid was pGEMM3, containing an engineered *Bam*HI site within *orf1* at its 5' end (Table 1). The  $Km^r$  cassette from pUC4K (30) was cloned in both orientations into the *Bam*HI site to form pGEMM3Km1 and pGEMM3Km2. Their *Kpn*I-*Mfe*I fragments spanning *orf1* (Fig. 2, pGPM3) were removed by band extraction from a gel of digests and used to replace the corresponding *Kpn*I-*Mfe*I fragment of pXQ3, forming pXQ3Km3 and pXQ3Km4. The entire IS*Ppu12*-*Km* insert of each was then excised with *Sph*I and *Xba*I and cloned into the *Sph*I and *Xba*I sites in the MCS of suicide vector pGP704, forming pGPTKm3 and pGPTKm4.

**Mobilization and transposition.** Plasmids pGPTKm1 to pGPTKm4 were transferred from S17-1 *λpir* into *P. putida* PRS2000 by filter mating, using a modification of the procedure described by Herrero et al. (9). Aliquots of stationary-phase cultures of donor and recipient were spotted together onto bacterial filters to give a cell/cell ratio of between 1:1 and 1:4. These were incubated at 30°C overnight on the surface of a nutrient agar plate. The mixed culture was resuspended in 100 mM phosphate (pH 7.5). For cell counts, the cell suspension and serial dilutions thereof were plated onto agar plates containing succinate plus kanamycin (for transconjugants), Isosensitest agar plus kanamycin (for donors plus transconjugants), or succinate alone (for recipients). The frequency of conjugation plus transposition was calculated as the ratio of transconjugants to donors.

**Overlap extension mutagenesis.** Four primers were designed for each of the four open reading frames (ORFs) (Table 2; Fig. 2): (i) an upstream and a downstream primer, each of which spanned a unique restriction site on plasmid pGPTKm1 or pGPTKm2 on either side of the ORF, and (ii) two complementary primers for each ORF which were partially complementary to a location towards the 5' end of each ORF but were designed to introduce a 7-bp deletion and a novel *Bam*HI site in the gene (Table 2). Using a two-stage PCR (11), fragments spanning the gene between the native upstream and downstream restriction sites but including the frameshift 7-bp deletion and *Bam*HI site were generated. These fragments were first cloned directly into pGEMTeasy vector. They were excised from this by using the upstream and downstream restriction sites, and these

fragments were used to replace the corresponding wild-type fragments from pGPTKm1 and pGPTKm2 (Fig. 2).

**Nucleotide sequence accession number.** The sequence of IS*Ppu12* as determined from the insert in pWW0-1216/2 has been deposited in GenBank with accession number AY128707.

## RESULTS

**The WR216 insert in *xylE*.** In order to extend the catabolic range of chlorobenzoate-degrading *Pseudomonas* sp. strain B13, strain WR216 was constructed by conjugative transfer of pWW0 into B13 followed by selection for acquisition of a novel phenotype, growth on 4-chlorobenzoate (24, 25). Previous comparison of the plasmid pWW0-1216 in WR216 with wild-type pWW0 had shown that, together with a deletion, it had acquired two novel insertions of about 3 to 3.5 kb, the critical one of which was in the *xylE* structural gene (for catechol 2,3-dioxygenase). This insert resulted in the polar inactivation of the downstream *meta* pathway operon (15), which was shown to be a biochemical prerequisite for the acquired 4-chlorobenzoate growth phenotype. The nature of either insert had never been explored previously.

We excised the insert in *xylE* from pWW0-1216, together with its flanking regions, on a 5.4-kb *Xho*I fragment and ligated it into pUC18, from which a 4.58-kb *Sal*I subfragment was recloned to form plasmid pXQ2 (Table 1). The complete nucleotide sequence of the fragment was determined. Comparison of the flanking regions with the sequence of the pWW0 *meta* pathway operon showed that the insertion had occurred after the 13th nucleotide of *xylE* (Fig. 1) and was sandwiched between an 8-bp direct repeat of bases 6 to 13 (CAA AGGTG) of *xylE* at both ends of the insertion.

The insertion in *xylE*, which is 3,372 bp in length and has been named IS*Ppu12*, has several main features (Fig. 1). At either end is an almost perfect (21 of 24) inverted repeat, GGGTA(A/T)(G/A)CGGATT(A/T)AATGGTTGAT; we have defined the two ends thus presented as IRR and IRL (Fig. 1), conforming with the nomenclature used by Weightman et al. (31). It also carries four ORFs (Table 3), one of which, from homology comparisons, clearly encodes a transposase (*tnpA*) and three of which are unrelated to known transposition genes. Transcribed in the same direction as *tnpA* and possibly forming a single transcriptional unit with it are *lspA*, encoding a lipoprotein signal peptidase homologue, and *orf1*, encoding a transporter or membrane protein homologue. Divergently transcribed from them is *orf2*, the putative product of which has similarities to regulator proteins.

**Comparison with pWW0 nucleotide sequence.** Alignment of the sequence of the 3,372-bp insert from pWW0-1216 with the complete nucleotide sequence of pWW0 showed the presence on the native plasmid of a single identical copy located between bases 84397 and 87768 within *Hind*III fragment E (6). This native copy, which is in the same orientation as the second copy in *xylE* of pWW0-1216, was not embedded between 8-bp directly repeated terminal sequences, the two flanking sequences being TCTTAAAT and AAAATTGA, respectively.

**Demonstration of transposition.** In order to demonstrate that IS*Ppu12* can transpose independently, derivatives which carried an inserted  $Km^r$  cassette as a phenotypic marker were constructed and then inserted into the suicide vector pGP704 (18). This plasmid is stable in *λpir* derivatives of *E. coli* and can

TABLE 3. Putative proteins encoded by *ISPpu12*

Gene designation	Putative function of gene product	Position in sequence <sup>a</sup> (position, gene designation [in pWW0 sequence] <sup>b</sup> )	Product size (residues, kDa)	Most similar gene product (species) (% amino acid identity, % similarity <sup>c</sup> ) (accession number) <sup>d</sup>
<i>orf2</i>	Possible regulatory protein	495–88 (87274–87681, pWW0164)	135, 15.4	PbrR protein ( <i>R. metallidurans</i> ) (44, 61) (CAC28872)
<i>orf1</i>	Possible membrane protein or transporter	591–1487 (87178–86282, pWW0163)	298, 31.2	Unknown protein ( <i>Actinobacillus actinomycetemcomitans</i> ) (97, 98) (NP_067562)
<i>IspA</i>	Lipoprotein signal peptide homologue	1491–2003 (86278–85766, <i>IspA</i> )	170, 18.8	Prelipoprotein signal peptidase ( <i>R. metallidurans</i> ) (42, 59) (CAC28875)
<i>tnpA</i>	Transposase	2100–3314 (85669–84455, <i>tnpA</i> )	404, 47.5	Putative transposase ( <i>P. stutzeri</i> ) (90, 92) (CAB_42636) (4)

<sup>a</sup> The sequence is numbered from IRR through to IRL as shown in Fig. 1.

<sup>b</sup> As annotated in GenBank (accession number AJ344068).

<sup>c</sup> As calculated following alignment of the amino acid sequences with ClustalX.

<sup>d</sup> The high degree of similarity between gene products and those of *S. marcescens* plasmid R471a is not included because they appear to be the same (see Discussion).

be transferred from these into recipient strains but will not replicate within them. In pGPTKm1 and pGPTKm2 (Table 1) the Km<sup>r</sup> cassette was inserted into the *KpnI* site in the intergenic region between the divergent *orf1* and *orf2* (Fig. 1) in the hope that, in this site, the cassette would not affect expression of any of the genes of *ISPpu12* and thus disable any transposition functions. These two plasmids were transferred from *E. coli* S17-1  $\lambda$ pir into *P. putida* PRS2000 in four separate experiments selecting for Km<sup>r</sup> transconjugants. In all of the matings a significant number were obtained (Table 4), and five were retained from each mating. Chromosomal digests of these were subject to electrophoresis and Southern blotted against a PCR probe generated from within the *orf1* gene of *ISPpu12*. One of the Km<sup>r</sup> transconjugants, PRS2000TK16, carried six distinct insertions, and at least 50% of those examined contained >1 insertion (Fig. 3). Fragments from *SalI* digests of the genomic DNA from PRS2000TK16 (Fig. 3, lane 9) were ligated into the *SalI* site of pUC18, and two Km<sup>r</sup> recombinant plasmids (pTK16S1 and pTK16S2) were isolated, each of which contained a different ~6-kb chromosomal fragment. These were subjected to single-strand sequencing outwards from each end of the *ISPpu12*-Km with primers complementary to regions about 100 bp inside the two terminal inverted repeats. The results clearly showed that the two termini of *ISPpu12*-Km were between coding sequences which showed similarities to other sequences in the databases but failed to show any significant length of nucleotide identity with any of the fragments from the partial sequence of *P. putida* PRS1

available in GenBank on 5 July 2002. In one transconjugant the two flanking sequences showed closest homology to the indoleacetamide hydrolase from *Rhizobium rhizogenes* (accession number QO9102) and the AMP nucleosidase from *Pseudomonas aeruginosa* (NP\_252659), respectively, and in the second transconjugant they showed closest homology to a LysR-like regulator protein from *Salmonella enterica* serovar Typhimurium (NP\_462508) and 1-cyclohexenyl carboxyl coenzyme A reductase from *Streptomyces collinus* (AAC44655). We were unable to confirm the exact chromosomal position from the limited data currently available from the partial sequence of *P. putida* PRS1 (PRS2000, A.3.12). In neither case was there an 8-bp direct repeat flanking the ends of the insertion sequence.

When pGPTKm1 and pGPTKm2 were transferred into *P. putida* PaW82 and *Pseudomonas* sp. strain WR1, Km<sup>r</sup> transconjugants were obtained at about the same frequencies (data not shown).

Two additional *ISPpu12*-Km constructs were made in which the Km<sup>r</sup> cassette was located within the 5' end of *orf1* (Fig. 2), and these were also placed in pGP704 as pGPTKm3 and pG-

TABLE 4. Transposition frequencies of *ISPpu12*-Km derivatives in *P. putida*

Donor plasmid (genotype)	Range of transposition frequency <sup>a</sup> (Km <sup>r</sup> transconjugants per donor)
pGPTKm1 .....	10 <sup>-4.2</sup> –10 <sup>-5.0</sup>
pGPTKm2 .....	10 <sup>-3.4</sup> –10 <sup>-4.7</sup>
pGPM1 ( $\Delta$ <i>tnpA</i> ) .....	No transconjugants
pGPM2 ( $\Delta$ <i>ispA</i> ) .....	10 <sup>-3.6</sup> –10 <sup>-5.3</sup>
pGPM3 ( $\Delta$ <i>orf1</i> ) .....	10 <sup>-4.2</sup> –10 <sup>-5.7</sup>
pPGM4 ( $\Delta$ <i>orf2</i> ) .....	10 <sup>-3.9</sup> –10 <sup>-4.6</sup>

<sup>a</sup> Each experiment was performed three or four times. The range is from the lowest to the highest in each series. Donor plasmids were in *E. coli* S-17  $\lambda$ pir, and the recipient was *P. putida* PRS2000.

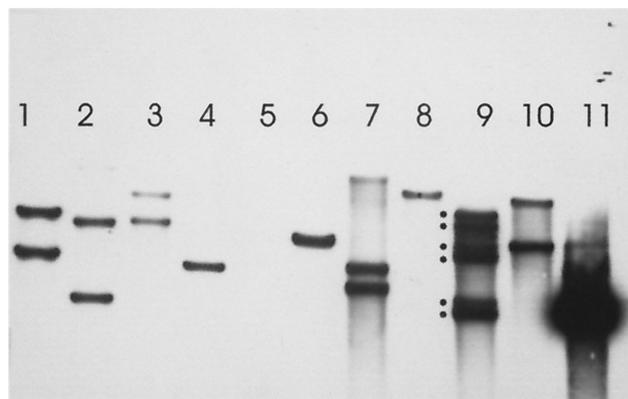


FIG. 3. Southern blots of *SalI*-digested genomic DNAs from *P. putida* PRS2000 transconjugants carrying copies of *ISPpu12*-Km. Lanes 1 to 4 and 6 to 10, nine different Km<sup>r</sup> transconjugants; lane 5, PRS2000 control; lane 11, *SalI*-digested pXQ3Km2 control. Note that the strain in lane 9 (PRS2000K16) has at least six copies of the IS element (marked with dots). The probe was a PCR-generated fragment spanning *orf1* (see Table 2).

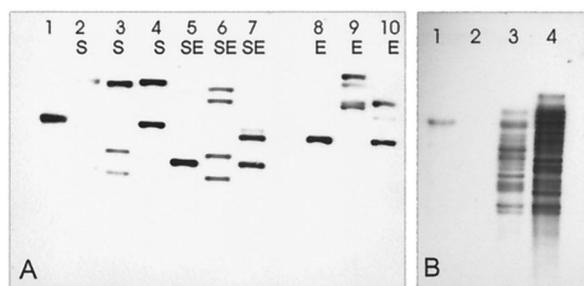


FIG. 4. Southern blots of digests of DNAs from *P. putida* mt-2 and *Pseudomonas* sp. strain B13 probed with the *orf1*-specific probe generated from IS*Ppu12*. (A) Plasmid DNAs. Lane 1, plasmid pXQ3Km2 control; lanes 2, 5, and 8, pWW0; lanes 3, 6, and 9, pWW0-1216-2; lanes 4, 7, and 10, pWW0-1216-3. Lanes S, digestion with *SalI*; lanes SE, digestion with *SalI* and *EcoRI*; lanes E, digestion with *EcoRI*. Neither *SalI* nor *EcoRI* cuts within IS*Ppu12*. (B) *SalI* digests of genomic DNAs. Lane 1, *P. putida* mt-2; lane 2, *Pseudomonas* sp. strain B13; lane 3, *Pseudomonas* sp. strain WR216-2; lane 4, *Pseudomonas* sp. strain WR216-3.

PTKm4 (Table 1). These plasmids transferred to *Pseudomonas* recipients PRS2000, PaW82, and WR1, producing Km<sup>r</sup> transconjugants with similar frequencies (data not shown), but no Southern blot analysis of transformants was carried out.

**Analysis of function of ORFs.** Using overlap extension PCR mutagenesis (10), we constructed four fragments, in each of which an internal 7-bp frameshift deletion coincident with a novel *Bam*HI site (for recognition purposes) had been engineered into the 5' end of one of the ORFs within IS*Ppu12*. These were individually ligated into the plasmid pGPTKm1 or pGPTKm2 on restriction fragments replacing the wild-type fragments (Fig. 2), to produce plasmids pGPM1 ( $\Delta$ *tnpA*), pGPM2 ( $\Delta$ *lspA*), pGPM3 ( $\Delta$ *orf1*), and pGPM4 ( $\Delta$ *orf2*) (Table 1). After digestion to confirm the presence of the introduced *Bam*HI site, the nucleotide sequence of each was determined on single strands to ensure that the expected mutations were incorporated. In the plasmids used, the PCR had introduced a few additional base changes as well as the designed deletion, but all were in the single gene being inactivated in each plasmid. S17-1 donors carrying each mutant plasmid were then mated with *P. putida* PRS2000 on filters under as near identical conditions (time and donor/recipient ratio) as was possible, and the frequency of transposition was determined as the ratio of Km<sup>r</sup> transconjugants to donor cells in the mating mixture. Only in the case of the *tnpA* knockout was transposition totally abolished, whereas inactivation of the other ORFs did not significantly change the frequency (Table 4).

**Analysis of pWW0-derived plasmids in *Pseudomonas* sp. strain B13 strains.** In the original analysis of pWW0-1216, the plasmid in *Pseudomonas* sp. strain WR216 (14, 15), comparison of its restriction digest with that of pWW0 showed that it contained two inserts of ~3.4 kb, one in *xylE* and the other in *Hind*III fragment C (coordinates 98347 to 115800). We therefore blotted digests of the plasmid from WR216, a clone of which we had retained for >20 years, against the PCR-generated *orf1* probe. We found that it contained four copies of IS*Ppu12* (Fig. 4A, lane 6): the two that we had originally identified (14); a copy in the native location in a fragment which contains part of *Hind*III fragment E, the existence of

which we had been unaware at that time; and a fourth copy, which has proved difficult to locate precisely from digests but is probably in the region between pWW0 coordinates 16000 and 22000. This fourth copy appears to have been acquired since 1982 during maintenance in our laboratory, as the plasmid digests are distinguishable from the pictures of digests that we have from that period. Unfortunately, there are no independent cultures of WR216 still in existence, since those retained by our collaborators in Germany proved to be no longer viable. To distinguish the present strain and its plasmid from the original, we have renamed them WR216-2 and pWW0-1216/2 (Table 1). We have also obtained a phenotypic homologue, WR216-3. This was recently constructed, as WR216 was, by plating the original parental strain WR211 onto 4-chlorobenzoate plates. Analysis of digests of its plasmid is complicated by the existence of both deletions and insertions, but it is clearly different from the plasmid in WR216, and Southern blots show that it contains only two copies of IS*Ppu12* (Fig. 4A); the location of one of these is in the native position (as in pWW0), but the location of the second has not been determined.

**Chromosomal copies of IS*Ppu12* in *Pseudomonas* hosts.** In order to check whether the ability of IS*Ppu12*-Km to transpose into the chromosome of PRS2000 was matched by the ability of the wild-type IS*Ppu12* to transpose into chromosomes, we blotted digests of genomic preparations of three derivatives of the pWW0 host *P. putida* mt-2 with the PCR-generated *orf1* probe. Wild-type mt-2 contained only one hybridizing fragment (Fig. 4B, lane 1), consistent with the single copy on pWW0, and the plasmid-free strains PaW82 and PaW340 contained no hybridizing fragment (data not shown). Wild-type *Pseudomonas* sp. strain B13 also contained no hybridizing DNA (Fig. 4B, lane 2). However, in extreme contrast, we compared blots of genomic DNAs from the B13-derived strains WR216 and WR216-3. In each of these there was a multiplicity of hybridizing bands, considerably more than were found in their TOL-derived plasmids, and the two patterns of blotting showed differences (Fig. 4B, lanes 3 and 4). This indicates that IS*Ppu12* has transposed not only within the TOL plasmid in these strains but also from the plasmid into the chromosome on multiple occasions. Both WR216 and WR216-3 were derived from WR211, but >20 years separated their constructions. The difference in the banding patterns between the two strains suggests differences in the transposition events which have taken place in the two separately maintained lines.

## DISCUSSION

Plasmids and transposons are tightly linked as important innovative elements in the evolution of bacterial genomes. The analysis of the nucleotide sequence of pWW0, one of only a limited number of catabolic plasmids to be sequenced, shows considerable evidence of a turbulent history of genetic rearrangements (8): a simple database comparison shows the presence of 13 transposase-like genes, gene fragments, or pseudogenes on the plasmid. In this paper we report a third active transposable element on the plasmid, the 3,372-bp insertion sequence IS*Ppu12*, without apparent phenotype, in addition to the large transposons Tn4651 and Tn4653 described previously (27–29). On the native pWW0, IS*Ppu12* is located between the two large transposases specific to Tn4651 and Tn4653, thus

TABLE 5. Genes surrounding *ISPpu12* on pWW0

Coordinates <sup>a</sup>	Gene or gene product designation
80113–79751	TnpC for Tn4651
83127–80122	TnpA for Tn4651
83233–83278	Right IR for Tn4651
83532–83296	Protein of unknown function, pWW0.154
83462–83785	Putative multidrug exporter, pWW0.155
83838–84170	Putative multidrug exporter, pWW0.156
84397–84420	IRL of <i>ISPpu12</i>
84421–87744	<i>ISPpu12</i>
87745–87768	IRR of <i>ISPpu12</i>
88308–87763	Terminated MerB protein
88402–88836	MerR homologue
89101–89661	TnpR for Tn4653
89665–92631	TnpA for Tn4653
92627–92664	Right IR for Tn4653

<sup>a</sup> As designated in the GenBank sequence AJ344068, the direction of transcription is indicated by the coordinates.

being within Tn4653 and in a region of only about 10 kb which contains all three of the pWW0 transposase genes demonstrated to be functional (Table 5).

As is also shown in the accompanying paper for a virtually identical insertion sequence and derived composite transposon, *DEH* (31), *ISPpu12* shows a remarkable ability to transpose in multiple copies. One pWW0-derived plasmid (pWW0-1216/2) carried four copies, only one of which (in *xylE*) appears to have been acquired in response to selective pressure. Multiple transposition of *ISPpu12* with an added Km<sup>r</sup> marker was also demonstrated from a suicide vector, where, in a single mating, more than half the transconjugants examined acquired between two and six chromosomal copies (Fig. 3). Remarkably, two strains derived from *Pseudomonas* sp. strain B13 which have been maintained with a TOL-derived plasmid for several decades contained a large number of chromosomal copies of the wild-type IS element (>10), whereas the wild-type B13 contained none (Fig. 4). Because of this property, it is surprising that *P. putida* mt-2, isolated and maintained as the natural host of pWW0 for an even longer period of time (>40 years), carries only the single plasmid-borne copy of *ISPpu12*, particularly since we have demonstrated that the Km-linked derivatives we have constructed in this work will transpose effectively within PaW82, a plasmid-free variant of strain mt-2. However, the facile transposability of *ISPpu12* appears to have been a persistent feature of the history of pWW0 and strain mt-2. In the very earliest stages of the codiscovery of the plasmid-encoded nature of the toluene-xylene genes, our culture of *P. putida* mt-2 (ATCC 33105) grew well on *m*-toluate by the plasmid-encoded catabolic pathway (33), whereas the strain described by Nakazawa and Yokota (TN1100) grew very poorly (20). The reason for this was discovered after the construction of pTN1, an RP4-pWW0 cointegrate plasmid which also supported only poor growth on *m*-toluate (21). Selection for improved and inducible growth resulted in a deletion of ~3 kb from pTN1 to form pTN2, the plasmid which subsequently served as the vehicle for studying the *xyl* catabolic genes in the Japanese laboratory (22). The nature of this deletion can be inferred from a second plasmid, pTN8, which was also selected from pTN1 for good growth on *m*-toluate but from which the *meta* pathway genes were expressed constitutively (12). The

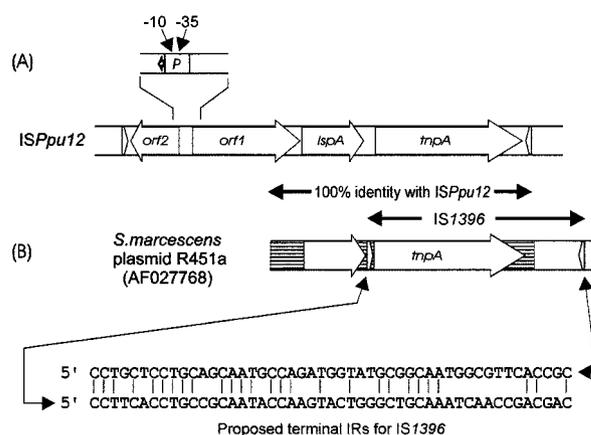


FIG. 5. Relationship between *ISPpu12* and other reported sequences. (A) Location of the 355 bp containing the constitutive promoter, firing from right to left, on pTN8 reported by Inouye et al. (12). (B) Postulated IS1396 from *S. marcescens* plasmid R471a (16). The map of IS1396 is drawn from the sequence under accession number AF027768 and is proposed to carry a *tnpA* gene between two terminal inverted repeats (IRs) (27 of 49 bp), shown on the map as blunt arrows with their sequences below. The area of the AF027768 sequence corresponding to 100% homology with *ISPpu12* is indicated by the hatched area spanning *tnpA* and running to the left to include an ORF identical to *ispA*. There is no similarity between *ISPpu12* and the nucleotide sequence on R451a to the right of IRL.

nucleotide sequence of a small, 355-bp region of pTN8 which determined the constitutivity was published (12) (accession number M12798). It contained a strong -10, -35 promoter, downstream of which was the 5' end of a gene claimed to be *xylE* (and still in the databases as such). Examined retrospectively, the entire 355-bp sequence is identical to part of *ISPpu12* (Fig. 5A) consisting of the intergenic region between *orf3* and *orf4*, where the promoter is located, plus the 3' end of *orf1* (86 bp) and the 5' end of *orf2* (174 bp), incorrectly identified as *xylE* (12, 19). pTN1 can therefore confidently be deduced to have carried a copy of *ISPpu12* located within the *meta* pathway operon upstream of *xylE* and blocking expression of the operon, causing the poor growth on *m*-toluate. Selection for spontaneous acquisition of faster growth on *m*-toluate resulted either in complete and precise excision to give pTN2 or in some smaller undetectable change to leave the *orf2* promoter firing through to *xylE* downstream and to give the constitutive phenotype of pTN8. For this to happen, this insertion in pTN1 must have been present in the TOL plasmid in the culture of *P. putida* mt-2 maintained in Nakazawa's laboratory but not in the strain that we were using, and also its exact position must have been further upstream of *xylE* and in the opposite orientation to that we later found in pWW0-1216 (cf. Fig. 1 and 5).

During Jeenes' doctoral studies of pWW0 in *Pseudomonas* sp. strain B13 (13), he constructed many derivatives of B13 in addition to those constructed in Knackmuss' laboratory (24, 25), and the presence of inserts of ~3 kb in novel locations within pWW0 were a frequent occurrence. Some of these plasmids, together with others generated in Broda's laboratory, were analyzed by restriction digestion and Southern blotting and, as we have shown here, were correctly deduced to be due

to internal transposition of a region around *Hind*III fragment E (17).

The results presented here show that *tnpA* is the only one of the four genes on IS*Ppu12* which affects its ability to transpose. The genetic structure of IS*Ppu12* indicates that *tnpA* might be the third gene of an operon downstream of *orf1* and *lspA*. If this is the case, then the expression of *tnpA* would not have been affected by our 7-bp deletion knockouts of the two upstream genes, which were deliberately designed not to have a polar effect. However, we also constructed two variants of IS*Ppu12* in which a Km<sup>r</sup> cassette was located within *orf1* in both orientations, which would certainly be expected to have a polar effect on expression of *tnpA* if it were part of the same transcription unit. The ease of transposability of these constructs suggests that *tnpA* is expressed independently of *lspA* and *orf1*.

The evidence from the work in Nakazawa's laboratory discussed above suggests that the promoter upstream of *orf2* is both active and constitutive, and it is interesting to speculate whether *orf2*, a regulatory gene homologue, has any functional role in IS*Ppu12* biology. The Km<sup>r</sup> cassette in two of the IS*Ppu12*-Km constructs, pGPTKm1 and pGPTKm2 (Fig. 1), would certainly have disrupted this promoter, and therefore expression of Orf2, since the *Kpn*I insertion site is directly between the -10 and -35 regions of the promoter (Fig. 5) and this does not affect the transposability. In addition, the similarity of transposition frequencies between the insertion sequence elements in which the Km<sup>r</sup> insertion is in the promoter and those with it alternatively located within *orf1* (pGPTKm3 and pGPTKm4), where the *orf2* promoter is not disrupted, strongly indicates that Orf2 is not involved in a regulatory role which affects transposition.

Whether *lspA*, *orf1*, or *orf2* confers a phenotype to IS*Ppu12*<sup>+</sup> cells remains a matter for speculation at this time. There are some possible links with genes involved in metal resistance. Two of the genes (*orf2* and *lspA*) have as their closest neighbors in the data banks genes involved in the *pbr* (lead resistance) gene cluster of *Ralstonia metallidurans* (Table 3), but otherwise the *pbr* cluster bears no similarity to IS*Ppu12*. Also, *orf2* shows similarity with some *merR* genes, and some of the genes adjacent to IS*Ppu12* in the complete pWW0 sequence also appear to be related to mercury resistance genes (Table 5). It is possible that the ancestry of IS*Ppu12* is in some way associated with metal resistance functions but that the presence of the genes within the transposable element is a fortuitous result of its history and has no biological significance. It is, however, worth noting that we have on a number of occasions attempted to determine whether pWW0 encodes metal resistance, by comparing the resistance of wild-type *P. putida* mt-2 with those of a number of its plasmid-free derivatives, including PaW82 and PaW340 (Table 1), and have failed to show any differences (data not presented). It seems, therefore, that any genes with homologies to resistance functions on pWW0 and, by implication, IS*Ppu12*, are no longer functional as such, if indeed they ever were.

Database searches show that the deduced amino acid sequence of the transposase is similar to those of other putative transposases in *Pseudomonas* strains described previously, such as IS*Pp1* from *Pseudomonas stutzeri* (4), but the overall structure of the complete insertion sequence is unlike any others,

apart from two which appear to be virtually identical. One of these is described in the accompanying paper and was found associated with dehalogenase catabolic genes in *Pseudomonas* (31). The other is reported in the databases as IS1396 from *Serratia marcescens* plasmid R471a (11, 16) (GenBank accession number AF027768). No evidence is available to demonstrate that it can actually transpose, but from sequence analysis only, IS1396 is reported as a 1,771-bp element with inverted repeats of 45 bp containing 21 mismatches. Its first 447 bp bears no relationship to IS*Ppu12*, but the remaining 1,325 bp is 100% identical and corresponds to IRL through the *tnpA* gene and *lspA* and into the 3' end of *orf1* (Fig. 5). However, the published sequence terminates at this point, and the postulated inverted repeat at this end of IS1396 corresponds to an internal part of *orf1*. In light of the sequences of IS*Ppu12* reported here and in the accompanying paper (31) and the experimental demonstration of its transposability, a more credible interpretation of the *S. marcescens* sequence is that its plasmid R471a carries a copy of IS*Ppu12* (or a very close relative) which has been incompletely sequenced and that the assigned terminal inverted repeats are the result of a fortuitous alignment of 24 of 45 bp, one within IS*Ppu12* and one upstream of it on plasmid R471a. If this is the correct explanation, it would indicate that IS*Ppu12* is a promiscuous element across a broad range of gram-negative genera and not exclusively confined to *Pseudomonas*.

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