Transposition of DEH, a Broad-Host-Range Transposon Flanked by ISPpu12, in Pseudomonas putida Is Associated with Genomic Rearrangements and Dehalogenase Gene Silencing

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Pseudomonas putida strain PP3 produces two hydrolytic dehalogenases encoded by dehI and dehII, which are members of different deh gene families. The 9.74-kb DEH transposon containing dehI and its cognate regulatory gene, dehR, was isolated from strain PP3 by using the TOL plasmid pWW0. DEH was fully sequenced and shown to have a composite transposon structure, within which dehI and dehR were divergently transcribed and were flanked on either side by 3.73-kb identical direct repeats. The flanking repeat unit, designated ISPpu12, had the structure of an insertion sequence in that it was bordered by 24-bp near-perfect inverted repeats and contained four open reading frames (ORFs), one of which was identified as tnpA, putatively encoding an ISL family transposase. A putative lipoprotein signal peptidase was encoded by an adjacent ORF, ispA, and the others, ISPpu12 orf1 and orf2, were tentatively identified as a truncated cation efflux transporter gene and a PbrR family regulator gene, respectively. The orf1-orf2 intergenic region contained an exact match with a previously described active, outward-orientated promoter, Pout. Transposition of DEH-ISPpu12 was investigated by cloning the whole transposon into a suicide plasmid donor, pAWT34, and transferring the construct to various recipients. In this way DEH-ISPpu12 was shown to transpose in a broad range of Proteobacteria. Transposition of ISPpu12 independently from DEH, and inverse transposition, whereby the vector DNA and ISPpu12 inserted into the target genome without the deh genes, were also observed to occur at high frequencies in P. putida PaW340. Transposition of a second DEH-ISPpu12 derivative introduced exogenously into P. putida PP3 via the suicide donor pAWT50 resulted in silencing of resident dehI and dehII genes in about 10% of transposition transconjugants and provided a genetic link between transposition of ISPpu12 and dehalogenase gene silencing. Database searches identified ISPpu12-related sequences in several bacterial species, predominantly associated with plasmids and xenobiotic degradative genes. The potential role of ISPpu12 in gene silencing and activation, as well as the adaptation of bacteria to degrade xenobiotic compounds, is discussed.

A key step in the biodegradation of halo-organic compounds is cleavage of the carbon-halogen bond. Many different enzymes generally referred to as dehalogenases catalyze this reaction; for example, different classes include hydrolytic, oxygenolytic, and reductive enzymes (21, 22). Dehalogenation of α-halocarboxylic acids such as the herbicide Dalapon (2,2-dichloropropionic acid) is catalyzed by one of the classes of hydrolytic dehalogenases that is best characterized, in that protein structures and mechanisms have been reported (31, 32, 36, 37) for the two distinct evolutionary families of these enzymes, the group I and group II deh products (17). There is preliminary evidence to suggest that other families of halocarboxylic acid dehalogenases exist (17, 23).

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The dehalogenase system of Pseudomonas putida strain PP3, a bacterium that was originally isolated from chemostat culture following selection on Dalapon (39), has been well studied. Strain PP3 produces two dehalogenases, DehI and DehII (55), which are encoded by genes of the group I and group II deh families, respectively (17). Thomas et al. (44, 45) showed that the dehI gene was carried on a mobile genetic element and gave it the general designation DEH, since it was found to vary in size following transposition into different plasmid targets. Thomas et al. (44) identified a hot spot for insertion of DEH into the TOL plasmid pWW0 (13, 57), and one such DEH element insertion was cloned and characterized (45) following its transposition from the PP3 genome to pWW0 and conjugal transfer of pWW0::DEH to another strain of P. putida. Thus, the DEH element was shown to carry the dehI gene immediately adjacent to dehR, which encoded a σ54-dependent activator (45, 46).

Several plasmid and chromosomal genes involved in the degradation of xenobiotic compounds are carried on catabolic transposons (43). Indeed, the genes encoding enzymes of the toluene catabolic pathway on pWW0 are carried on two nested Tn3-like elements, Tn4653 and Tn4653 (49, 50). Such catabolic transposons and insertion sequence elements have been strongly implicated in the evolution of catabolic functions associated with adaptation of bacteria to degrade xenobiotic
compounds (52). Evidence has also been reported to suggest that activation and/or silencing of such genes is associated with insertion sequence elements (11). Our previous studies have shown that spontaneous mutants of strain PP3 resistant to inhibition by dichloroacetic acid (DCA), a toxic α-halocarboxylic acid, were selected at high frequencies (41, 54) and that in most of these mutants either one or both of the dehalogenase genes were silenced or lost. In some mutants silenced dehI genes were reactivated, and it was suggested that silencing and reactivation of dehI and dehII genes, associated with DCA resistance, might involve movement of mobile DNA in strain PP3 (41); however, no genetic link between DEH transposition and dehalogenase gene switching was established. Mobile elements associated with other dehalogenase-producing bacteria have been reported (25, 26, 53), as has preliminary evidence for dehalogenase gene silencing in a Rhizobium species (29).

The aim of the present study was to investigate in more detail the structure and function of the DEH transposon from strain PP3, carrying dehI and dehII. Here we report the full sequence of the DEH element, including ISPpu12, and present results from experiments investigating its transposition and associated effects.

MATERIALS AND METHODS

Bacterial strains and plasmids. Escherichia coli strain IM107 was used for routine plasmid maintenance (59). E. coli strain S17-1 (40) was used as the donor strain in mating experiments to transfer DEH-ISPpu12 to plasmids pAWT34 and pAWT50 and was kindly provided by Reinhard Simon. P. putida strains PaW1 and PaW340 (strain mt-2 derivatives) (3), UWC1 (also an mt-2 derivative) (34), PPI (42), and PPI-KS1 (a spontaneous rifampin-resistant derivative of strain PP3) were used as recipients in mating experiments. Plasmid pAWT6, a derivative of pHG327 containing the DEH element from a TOL plasmid pWW0 recombinant, was described previously (44, 45) and was ligated after partial digestion with EcoRI into pSUP202, a suicide plasmid donor constructed by Simon et al. (40) and carrying a tetracycline resistance (Tcr) element, including ISPu1 site in dehI (dehIR), producing a dehalogenase knockout and an alternative selective marker for transposition.

Media and growth conditions. E. coli and P. putida cultures were routinely grown in Luria-Bertani medium (1% tryptone, 0.5% yeast extract, 1% NaCl) and on nutrient agar (Difco), with appropriate antibiotic, vitamin, and antibiotic additions, at 37 and 30°C, respectively. SBS (42) minimal medium was also used, with the additional of filter-sterilized 14 mM 2-chloropropionic acid (2MCPA) (pH 7) or 10 mM sodium succinate for growth of prototrophic strains. Solid media were prepared with the addition of 1.5% agar (Difco).

Antibiotics and amino acids were sterilized by filtration through a 0.2-μm pore-size cellulose acetate membrane (Whatman) and added to autoclaved media when cooled to 55°C.

Transposition of DEH suicide plasmid donation by filter matings. E. coli strain S17-1 (40) was transformed with plasmids pAWT34 and pAWT50 by a rapid transformation protocol (14). E. coli strain S17-1 has the transfer functions of plasmid RP4 incorporated into the chromosome (40), allowing the mobilization of plasmids without transfer of a second conjugative plasmid. Conjugative transfer of pAWT34 into P. putida strain PAW340 and of pAWT50 into strain PP3 was achieved by filter mating. Overnight cultures of the donor and recipient organisms were concentrated 10-fold and mixed in equal proportions, and 50 μl of each mixture was deposited on SBS-agarose blocks from overnight cultures of P. putida, and plated at an appropriate dilution onto selective agar. Any plasmids transferred to strain PAW340 were selected on 2MCPA-SBS minimal medium supplemented with tetracycline (0.1 mM) and 500 μg of streptomycin (for selection of PAW340 strains containing DEH) per ml. Colonies were then screened for replica plating onto nutrient agar (NA) containing 50 μg of tetracycline per ml for absence of Tc, to identify true transposition transconjugants that did not contain pSUP202 vector. The pSUP202 vector contained the Tcr of NA with 50 μg of tetracycline per ml and 500 μg of streptomycin per ml (the Tc of pAWT34 was derived from the pSUP202 vector (40)) and screened for inability to grow on 2MCPA (i.e., absence of dehI) to identify inverse (vector) transposition transconjugants. The matings to transfer pAWT50 to strain PP3 were plated onto Pseudomonas aeruginosa selective agar (PSA) (Oxoid) containing 50 μg of kanamycin per ml (DEH::Km transposition) and on PSA containing 40 μg of tetracycline per ml (inverse transposition), with screening to eliminate double-resistant (Km’ Tc’) colonies. Transconjugants from the latter matings were tested for resistance to DCA on SBS-succinate minimal medium containing 50 mM DCA.

DNA isolation, purification, and manipulation. Cultures used for isolation of total DNA were grown overnight in Luria-Bertani medium. A 1.5-ml portion of culture was harvested, and the cells were washed in 1 ml of TES buffer (50 mM Tris-HCl, 5 mM EDTA, 50 mM NaCl). Cells were then resuspended in 500 μl of 25% sucrose in 50 mM Tris-HCl (pH 8), and 100 μl of lysozyme (50 mg/ml in 0.25 M EDTA [pH 8]) was added. Tubes were incubated on ice for 30 min. Lysis was completed by the addition of 25 μl of 20% SDS and 25 μl of proteinase K (25 mg/ml) (Roche). The lysates were transferred to dialysis tubing (Fisher) and dialyzed overnight against 50 mM EDTA (pH 8)-0.5% SDS at 56°C.

At least 50 ml of dialysis solution was allowed for each cell lysis. After overnight incubation, the EDTA concentration of the lyses was reduced by replacing the dialysis solution with TE buffer (10 mM Tris-HCl, 1 mM EDTA) and gently stirring at room temperature for at least 2 h. Lysates were then removed from the dialysis tubing and gently extracted twice with phenol-chloroform (50:50 phenol, 48% chloroform, 2% (w/v) sodium alcohol) in order to remove proteinase K. Phenol was removed by two extractions with water-saturated ether. DNA was precipitated by the addition of 2 volumes of absolute ethanol (~20°C) and gentle mixing. DNA was immediately harvested by centrifugation, washed in 1 ml of 70% ethanol, dried under vacuum, and dissolved in 200 μl of sterile deionized water.

Genomic DNA for pulsed-field gel electrophoresis (PFGE) was prepared in agarose blocks from overnight cultures of P. putida, as follows. Five hundred microliters of culture was harvested by centrifugation, washed in SE (75 mM NaCl, 25 mM EDTA, pH 8), and finally resuspended in 500 μl of agarose buffer (10 mM Tris-HCl, 10 mM MgCl₂, 0.1 mM EDTA, pH 7.5). The cell suspension was added to agarose tubes containing 0.5 mg/ml of lysozyme per ml and incubated overnight at 37°C. The mix was transferred to an agarose block mold (Bio-Rad) and allowed to set at 4°C for 20 to 30 min. Blocks were removed and placed in a partitioned petri dish to separate them. Lysis solution (100 mM EDTA, 1% SDS, 1 μg/ml of lysozyme per ml in 10 mM Tris-HCl, pH 8.0) was added to 2 ml per block and incubated overnight at 37°C. The lysis solution was removed carefully, and the blocks were then washed in TE buffer. Each block was incubated in protein digestion solution (50 mM EDTA [pH 9.5], 1% SDS, 0.5 mg of proteinase K per ml) for 48 h at 56°C and washed again in TE buffer. Proteinase K was inactivated by incubating each block in 2 ml of TE buffer containing 1 mM phenylmethylsulfonyl fluoride overnight. Following a final wash for at least 1 h in TE buffer, the blocks were stored at 4°C in fresh TE buffer until required.

DNA digestion with restriction endonucleases was carried out for at least 3 h, using an excess of enzyme and the manufacturer’s recommended buffer and temperature. Restriction endonucleases were purchased from Promega, Roche, and Amersham-Pharmacia.

Standard agarose gel electrophoresis of DNA fragments was carried out using 0.8% agarose (Ultrapure grade; Life Technologies) and Tris-borate-EDTA buffer as described by Sambrook et al. (38). For PFGE, slices of the agarose blocks (ca. 1/5 of the total) containing genomic DNA were equilibrated on ice for 1 h in Spel restriction endonuclease buffer and then overnight at 37°C in 200 μl of fresh buffer containing 20 U of Spel. Following digestion, gel slices were placed in the wells of a 1.5% agarose gel (in 0.5× TBE) and sealed with leaving-empty agarose. PFGE was carried out at 200 V at 18°C with the switch time set to ramp from 3 to 15 s over the first 5 h and from 1 to 33 s over the next 19 h. After staining with ethidium bromide and destaining in H₂O₂ for 30
min. DNA in the gels was depurinated by incubation at 37°C in 0.05 M HCl for 30 min prior to Southern hybridization.

Southern hybridizations. Following electrophoresis and staining, DNA in agarose gels was denatured by gentle agitation of the gel in 0.5 M NaOH-1.5 M NaCl for 20 min. The denaturing solution was then replaced, and agitation was continued for another 20 min. The gel was then rinsed in distilled water and neutralized for 30 min in 0.5 M Tris-HCl (pH 7.5)–2 M NaCl, with a change of solution after 15 min. DNA was transferred overnight to a Hybond-N nylon membrane (Amersham-Pharmacia) by using the double squash blot technique (38). DNA was fixed to the damp membrane by 3 min of exposure on a 310-nm transilluminator. Membranes were air dried and stored at room temperature.

DNA hybridizations were performed with the digoxigenin (DIG) nonradioactive labeling system (Roche). As indicated in Fig. 1, a 2.56-kb Smal fragment and a 1.23-kb BsrEI fragment were used as probes to detect transposition of dehI-dehRf and ISPpu12, respectively. Inverse (vector) transposition was detected using EcoRI-linearized pSUP202 as a probe. Restriction fragments used as probes were gel purified with GeneClean II resin (Bio Inc.) according to the manufacturer’s recommended protocol. Probes were separately labeled with DIG by using a random multiprime labeling system, following the protocol of the manufacturer (Roche). A 125-bp DIG gene probe was also used in some experiments and was DIG labeled using a PCR protocol (30) with primers dehIf (5'-GCT TGT ACG CTG CAG GAT TCG AT) and dehIr (5'-CGC ATC TGC ATG CTT CTA CT). PCRs were carried out with pAWT6 as the template and standard concentrations of reagents under the following cycling conditions: denaturation, 95°C for 30 s; annealing, 55°C for 30 s; and extension, 72°C for 60 s (32 cycles). The probe was gel purified and stored at 4°C until required.

Hybridizations were carried out at 42°C, using a hybridization solution containing 5× SSC (0.75 M NaCl plus 0.0825 M sodium citrate), 50% formamide, 2% skim milk powder (blocking agent), 0.1% N-lauryl sulfate, and 0.02% SDS. After hybridization, high-stringency washes of the membranes were carried out. Hybridizing bands were developed in accordance with the instructions of the manufacturer (Roche), using a 1:10,000 dilution of anti-DIG alkaline phosphatase conjugate and AMPPD as the enzyme substrate. X-ray film was exposed, at room temperature, for 1 to 12 h and developed using FX-40 developer (Kodak) and Hypam fixer (Ilford).

DNA sequencing and analysis. The strategy for DEH transposon sequencing involved both shotgun cloning of purified restriction fragments from plasmid pAWT6 into pUC-based vectors (59) and primer walking on PCR fragments amplified and cloned from P. putida PPS. At least triple coverage was achieved on both strands. The following software was used for sequence assembly and analysis: DNAsis (Hitachi), Artemis (www.sanger.ac.uk/Software/Artemis/v4), and various other programs available from the Sanger Centre (www.sanger.ac.uk), the National Center for Biotechnology Information (www.ncbi.nlm.nih .gov), and the European Bioinformatics Institute (www.ebi.ac.uk). Classification of ISPpu12 was done with reference to the insertion sequence database (www-is .biotoul.fr/is.html) (33) with the kind assistance of Jacques Mahillon. Motifs associated with the σ45 promoter were identified by using SEQSCAN (www.bmb .psu.edu/seqscan) with reference to the work of Barrios et al. (2).

Nucleotide accession number. The EMBL accession number of the complete DEH sequence is AY138113.

RESULTS

Structure of the DEH element and its flanking insertion sequence, ISPpu12. The structure of the 9.74-kb DEH element originally cloned from pWWW0:DEH (44, 45) was determined by complete sequence analysis (Fig. 1). The full sequence of dehI adjacent to and transcribed divergently from dehRf (its cognate regulatory gene) is reported for the first time; however, the sequence of dehRI was previously reported (46), as were results showing that expression of dehI in the DEH element was not observed in an rpoN mutant of P. putida (45). A putative −10/−35 promoter for dehRI and a −12/−24 promoter for dehI were identified, the presence of the latter being consistent with this σ54 (RpoN) dependence. In addition, sequence analysis of the intergenic region between dehRI and dehI identified other motifs, specifically, a putative integration host factor-binding region and two upstream-activating sequences, which are commonly associated with σ45-dependent promoters (2, 58).

The dehI-dehRI region had one copy of a 3.37-kb element on either side, directly repeated (Fig. 1). This element, designated ISPpu12, contained four open reading frames (ORFs) and had the structure of a large insertion sequence, in that one of the ORFs encoded a putative transposase and it was flanked by 24-bp imperfect inverted repeats (IRs) (Fig. 2B). Highly significant nucleotide matches (>90% sequence identity) were uncovered between ISPpu12 and regions (0.8 to 3.37 kb) of the following plasmids: pWW0 from P. putida mt-2 (13), R471a from Serratia marcescens (28), pVT745 from Actinobacillus actinomycetemcomitans (12), and pLEM from Pasteurella multocida (35). The following unrelated xenobiotic degradative gene clusters in Pseudomonas species also contained flanking sequences that showed >90% sequence identity to regions of ISPpu12: ortho-halobenzoate, ohb (48); alkylbenzene, xyl (5); chlorocatechol, cfc (16); and napthalene, nah (7). The intergenic region between the hypothetically divergently tran-
FIG. 2. (A) Hypothetical schematic pathway by which DEH-ISpu12 recombined with resident copy of ISpu12 on pWW0 during plasmid rescue of DEH-ISpu12 from P. putida strain PP3 (44). The precise sites of the proposed recombination between the different ISpu12 elements, indicated by X, are not known. (B) Nucleotide alignment of ISpu12 IRs IRL and IRR. (C) Nucleotide alignment of ISpu12 from strain PP3 and ISpu12 from pWW0 (56) in the intergenic orf1-orf2 region and within orf2, highlighting (in grey) the sequence differences around the −35/−10 Pout promoter (shown in boldface) (18). The position of the putative start to orf2 is indicated in boldface and underlined, and the derived amino acid sequences for ISpu12 from PP3 and from pWW0 are given above and below their respective nucleotide sequences. The unique diagnostic PstI site in ISpu12 from PP3 is in italics and underlined.
The putative transposase gene, *tnpA*, of *ISPpu12* was identical to that reported by Kulaeva et al. (28) for a 1,771-bp element designated IS1396, but only one of the IRs proposed for IS1396 was found in the *DEH* sequence. On the basis of alignments of the TnpA-derived sequence with those of other transposases and further analysis (J. Mahillon, personal communication), *ISPpu12* was tentatively assigned to the ISL3 family according to the classification of Mahillon and Chandler (33).

As indicated in Fig. 1, the three ORFs upstream of *tnpA* in *ISPpu12* encoded putative products with high levels of predicted amino acid sequence similarity to the following gene products: a lipoprotein signal peptidase (44% similarity over the ORF with LspA from *P. fluorescens* [19, 20]), a putative heavy-metal-associated cation efflux transporter (truncated, Orf1 [1, 51]), and a heavy metal resistance regulator (Orf2, >95% similarity to PbrR family regulator helix-turn-helix N-terminal domain [15]). None of these three ORFs is commonly contained within insertion sequence elements, nor is any of them thought to be directly associated directly with transposition. It was noted that these ORFs each had homologues in *Ralstonia metallidurans* strain CH34 (6), as follows: PbrC (LspA), CzcD (Orf1), and PbrR (Orf2). All of these homologues are associated with other insertion sequence elements in that organism.

**Origins of *ISPpu12* and *ISPpu12R* elements in transposon *DEH*.** Comparison of *ISPpu12* and *ISPpu12R* (Fig. 1) in the *DEH* element cloned from pWW0::*DEH* revealed some minor sequence differences in the intergenic region between *orf1* and *orf2* and within *orf2* (Fig. 2C), comprising 15 nucleotide positions downstream and 1 upstream from promoter Pout. In order to establish whether the structure of *DEH* and the sequences of its flanking *ISPpu12* elements were the same in *P. putida* strain PP3 as in the pWW0::*DEH* derivative, PCR cloning was used to isolate separately *ISPpu12L* and *ISPpu12R* directly from strain PP3. PCR restriction mapping and sequencing confirmed that the *DEH* element in strain PP3 was flanked by two identical copies of *ISPpu12*. Southern hybridization experiments showed that strain PP3 contained only one copy of the *dehI* gene and one copy of *DEH* (L. L. Lee and A. J. Weightman, unpublished results). However, restriction mapping results experimentally confirmed that one copy of *ISPpu12* in pWW0::*DEH* was slightly different from the *ISPpu12* elements in strain PP3, in that the latter contained an extra *PstI* site located within *orf2* (Fig. 2C).

This was further investigated by high-stringency Southern hybridization experiments using the *ISPpu12* probe (derived from pWW0::*DEH*), which showed that pWW0 contained a very similar element within the *EcoRI*-G restriction fragment of that plasmid (47), the region that Thomas et al. (44) had shown to be a hot spot for *DEH* insertion. Comparison of the *ISPpu12R* sequence derived from pWW0::*DEH* with the copy of *ISPpu12* resident in pWW0 (13) confirmed the hybridization results and showed an exact match. Therefore, it is proposed that the *DEH* element originally described by Thomas et al. (44, 45) was formed by recombination between the *ISPpu12* elements of *DEH* in strain PP3 and the *ISPpu12* element resident in pWW0 (Fig. 2A). However the specific site of recombination could not be identified because of the extensive homology between *ISPpu12* in PP3 and that in pWW0. Although there were these minor sequence differences between *ISPpu12* in strain PP3 and *ISPpu12* in pWW0, the elements appeared to be identical with respect to all of their major features (Fig. 1), including IRs, ORF sequences, and the presence of an active promoter sequence located upstream from *orf2* (18, 56).

**Transposition of *DEH* and *ISPpu12*.** The *DEH* element from plasmid pWW0::*DEH* was recloned from plasmid pAWT6 (45) by using the suicide donor plasmid pSUP202 (40) to form plasmid pAWT34, and *E. coli* strain S17-1(pAWT34) was used as a transposon donor in 6-h filter matings.

In the first series of filter mating experiments, plasmid pAWT34 was transferred to the recipient *P. putida* strain PaW340 and transconjugants were selected on the following media: (i) SBS minimal medium containing tryptophan, streptomycin, and 2MCPA to select for true (*DEH*) transposition, with screening for absence of Tc<sup>+</sup> associated with the pSUP202 vector; and (ii) NA containing streptomycin and tetracycline to select for inverse (vector) transposition, with screening for inability to utilize 2MCPA (i.e., absence of *dehI*). The use of a suicide plasmid donation system to introduce *DEH* into the recipients prevented the calculation of transposition frequencies per plasmid transfer; however, the combined transfer-transposition frequencies observed were remarkably high. For example, after a 6-h filter mating, approximately 6% of all potential strain PaW340 recipients were able to grow on 2MCPA-containing minimal medium, indicating that true transposition had occurred. Furthermore approximately 2.5% of all potential recipients were Tc<sup>+</sup>, indicating that inverse (vector) transposition had occurred at high frequency. Unexpectedly, it was found that a high proportion (43%) of unscreened transconjugants selected on 2MCPA were also Tc<sup>+</sup> and, conversely, 52% of unscreened transconjugants selected as Tc<sup>-</sup> were also able to grow on 2MCPA-containing minimal medium. It is possible that such transconjugants resulted from a single recombination and integration of pAWT34 into the recipient chromosome; however, hybridization results with probes to detect true and inverse transposition products (see below) indicated that multiple independent transposition events had occurred in these strains (47).

Figures 3 and 4 show the results from Southern hybridizations using three probes (the *dehI-dehIR*, and *ISPpu12* probes shown in Fig. 1 and a pSUP202 vector probe) against *EcoRI* and *XhoI*-digested genomic DNAs from transconjugants selected from independent filter matings. None of the probes hybridized with DNA from the recipient strain PaW340, and the hybridization patterns in transconjugants confirmed that *ISPpu12* was associated with all true (Fig. 3) and inverse (Fig. 4) transposition events. Figure 3A shows that the copy number of *DEH* in transconjugants selected for true transposition, estimated by halving the total number of hybridizing *XhoI* fragments (*DEH* contained one *XhoI* site located within the *dehI-dehIR* probe [Fig. 1]) varied from 1 (Fig. 3A, lane 8) to >7 (lanes 3, 4, 5, and 6). Figure 3B shows that independent transposition of *ISPpu12* was observed in a high proportion (at least 6 of 10) of these transconjugants and that in half (3 of 6) of these >1 additional independent copy of *ISPpu12* was evident. It should be noted that these transconjugants were selected as...
Tc<sup>c</sup>, and none hybridized with the pSUP202 vector probe (results not shown). Conversely, none of the transconjugants shown in Fig. 4, selected for inverse transposition and screened for their inability to grow on 2MCPA (i.e., lack of dehalogenase activity), hybridized with the <i>dehI-dehRI</i> probe (results not shown). The characteristic features of inverse transposition transconjugants were that multiple copies of the vector-containing transposition product were not observed but independent transposition of IS<sub>Ppu12</sub> still occurred (Fig. 4B).

Southern hybridizations of Tc<sup>c</sup>, 2MCPA-positive transconjugants (i.e., those originally selected on 2MCPA and subsequently found to be Tc<sup>c</sup>) showed a pattern of results similar to those illustrated in Fig. 3, with multiple insertions of <i>DEH-IS<sub>Ppu12</sub></i> (with IS<sub>Ppu12</sub> also transposing independently from

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**FIG. 3.** Southern hybridization showing true transposition of <i>DEH-IS<sub>Ppu12</sub></i> from pAWT34 into the chromosome of <i>P. putida</i> PaW340. Ten independent transposition transconjugants were selected on 2MCPA-SBS medium from 6-h filter matings between the donor <i>E. coli</i> S17-1(pAWT34) and recipient PaW340. (A) Genomic DNAs from strains PaW340 T1 to T10 digested with <i>XhoI</i> (lanes 1 to 10, respectively) and <i>EcoRI</i> (lanes 11 to 20, respectively), blotted, and hybridized with the DIG-labeled <i>dehI-dehRI</i> probe (Fig. 1). Lane 21, control DNA from strain PaW340 digested with <i>EcoRI</i>. (B) Replicate Southern blot hybridized with the <i>IS<sub>Ppu12</sub></i> probe (Fig. 1). Circles are placed alongside <i>XhoI</i> DNA fragments hybridizing only with DIG-labeled <i>IS<sub>Ppu12</sub></i>, indicating transposition of this element independently of <i>DEH</i>. All strains were Tc<sup>c</sup>, and none hybridized with the pSUP202 vector probe (results not shown).

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**FIG. 4.** Southern hybridization showing inverse transposition of <i>DEH-IS<sub>Ppu12</sub></i> from pAWT34 into the chromosome of <i>P. putida</i> PaW340. Ten independent transposition transconjugants were selected on NA-tetracycline medium from 6-h filter matings between the donor <i>E. coli</i> S17-1(pAWT34) and recipient PaW340. (A) Genomic DNAs from strains PaW340 I1 to I10 digested with <i>XhoI</i> (lanes 1 to 10, respectively), blotted, and hybridized with the DIG-labeled pSUP202 probe. (B) Replicate Southern blot hybridized with the DIG-labeled <i>IS<sub>Ppu12</sub></i> probe (Fig. 1). Circles are placed alongside DNA fragments hybridizing only with <i>IS<sub>Ppu12</sub></i>. Dehalogenase activity was not detected in any of these strains, nor did they hybridize with the <i>dehI-dehRI</i> probe (results not shown), and none was able to grow on 2MCPA.
DEH) and single-copy insertions of the inverse product associated with Tc'. Thus, it was evident that these transconjugants had not simply arisen from whole plasmid pAWT34 integration into the recipient genome. In all transposition transconjugants tested, DEH-IS\textit{Ppu12} transposons could be rescued in \textit{P. putida} UW1C by mating-out experiments with exogenously introduced pWW0 and appropriate selection, i.e., growth on 2MCPA for true transposition products and Tc' for inverse transposition products. Frequencies of transposition in these rescue experiments ranged from $5 \times 10^{-4}$ to $1.5 \times 10^{-6}$ per plasmid pWW0 transfer but did not correlate with IS\textit{Ppu12} copy number in the donor strain.

The host range of IS\textit{Ppu12} was determined by using pWW34 and selection for Tc' (inverse) transposition where \textit{DEH} (true) transposition could not be selected directly. In this way, IS\textit{Ppu12} transposition was observed in the following species, representing the \textalpha{}, \textbeta{}, and \textgamm{} subdivisions of the \textit{Proteobacteria}: \textit{A. tumefaciens}, \textit{B. cepacia}, \textit{C. tertagudina}, \textit{R. eutropha}, \textit{Acineto bacter sp.}, \textit{P. aeruginosa}, and \textit{P. putida}.

\textbf{Gross genomic rearrangements in \textit{P. putida} PaW34.} PFGE was used to investigate the distribution of multiple copies of \textit{DEH} in transconjugants selected on 2MCPA. Figure 5B shows a Southern hybridization of \textit{SpeI}-digested genomic DNA from selected true-transposition PaW34 transconjugants, using a PCR-generated \textit{dehI} probe. Since \textit{DEH} contained no \textit{SpeI} site, the presence of multiple large hybridizing fragments evident in most transconjugants indicated that the element had transposed promiscuously into different loci on the PaW340 chromosome, and in most cases multiple transposition events had occurred during the selection process. Unexpectedly, the \textit{SpeI} digest patterns of the transconjugants showed polymorphisms (Fig. 5A), to such an extent that no two had the same \textit{SpeI} restriction fragment length polymorphism profile and all were different from that of the PaW340 recipient. The observation that several large (\textgtr{}100-kb) \textit{SpeI} fragments were affected suggested that \textit{DEH} transposition was associated with gross genomic rearrangements of the PaW340 chromosome. No \textit{dehI}-hybridizing fragment was seen in one of the transconjugants (Fig. 5B, lane 3), because the \textit{DEH} element had inserted into an \textit{SpeI} fragment that was too small (i.e., \textless{}35 kb) to be visible on the gel after PFGE under the conditions used.

\textbf{Transposition of \textit{DEH} associated with mutagenesis and dehalogenase gene silencing in \textit{P. putida} PP3.} A derivative of plasmid pAWT34 containing a Km' \textomega{} interposon (10), inserted to knock out \textit{dehI}, and thereby prevent activation of \textit{dehI} expression, was constructed and designated pAWT50. This suicide donor plasmid derivative was used to transfer \textit{DEH}::\textomega{}-Km to a rifampin-resistant derivative of \textit{P. putida} strain PP3, designated PP3-KS1. Strain PP3-KS1 transconjugants in which true transposition (insertion of \textit{DEH}::\textomega{}-Km) had occurred were selected on PSA-kanamycin, and inverse (vector) transposition transconjugants were selected on PSA-tetracycline. The results summarized in Table 1 confirmed that IS\textit{Ppu12} was associated with true and inverse transposition. The observation that ca. 3% of transconjugants were putative auxotrophs (i.e., unable to grow on succinate-containing minimal medium) suggests that IS\textit{Ppu12} and \textit{DEH} derivatives were mutagenic, as is frequently observed with insertion sequence elements and transposons. Five of these putative auxotrophs were further characterized by auxanography (8) and were shown to contain different mutations in one of the following independent amino acid biosynthetic pathways: methionine (strain PP3-KS2), cysteine (PP3-KS3), tryptophan (PP3-KS4 and PP3-KS6), and leucine (PP3-KS5).

Mutations causing DCA resistance (ca. 20%) and inability to utilize 2MCPA (2MCPA-negative mutants constituted ca. 5% of transconjugants, and all were DCA resistant as expected) were detected at high frequencies in true- and inverse-transposition transconjugants (Table 1). Both of these types of mutations are known to be associated with silencing of the dehalogenase genes in strain PP3, in that it was previously shown that mutants unable to utilize 2MCPA expressed neither \textit{dehI} nor \textit{dehII}, and most DCA-resistant mutants expressed either only one or neither of these genes (41, 54). Southern hybridizations of inverse (Km' Tc')-transposition transconjugants of strain PP3 that had become 2MCPA negative (i.e., produced no dehalogenase) with a \textit{dehI} probe showed that they all still
TABLE 1. Transposition of DEH-ISPpu12 transferred exogenously to P. putida strain PP3 by using suicide donor plasmid pAWT50

<table>
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<tr>
<th>Selected transposition transconjugant class (mode of detection)</th>
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<td></td>
<td>True transposition</td>
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</tr>
<tr>
<td>Inverse transposition (Tc&lt;sup&gt;b&lt;/sup&gt;)</td>
<td>54</td>
</tr>
<tr>
<td>Autotrophic (unable to grow on succinate-SBS)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3</td>
</tr>
<tr>
<td>DCA resistant (growth on succinate-DCA-SBS medium)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>22</td>
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<tr>
<td>Silenced dehI and dehII (2MCPA negative, unable to grow on SBS-2MCPA medium)</td>
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<sup>a</sup> Filter matings (6 h) were carried out as described in Materials and Methods; the donor was E. coli S17-1(pAWT50), and the recipient was P. putida PP3. True- and inverse-transposition transconjugants were selected independently on PSA kanamycin and PSA-tetracycline media, respectively. Five hundred transconjugants from three independent matings were isolated, purified, and characterized by patching onto the appropriate media to detect each of the specified classes of transconjugant.

<sup>b</sup> Ten auxotrophs were further characterized by auxanography (see text).

<sup>c</sup> More than 90% of DCA-resistant mutants of PP3 do not express dehI and/or dehII (54; L. L. Lee and A. J. Weightman, unpublished results).

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**DISCUSSION**

The results reported here show that the DEH element in P. putida PP3 is a composite transposon on which the dehalogenase gene dehI and its cognate regulatory gene, dehRI, are located side by side and flanked by identical direct repeats of an insertion sequence, ISPPu12. Transposon Tn5 and insertion- 

The main evidence suggesting that DEH-ISPpu12 is a composite transposon may be summarized as follows: (i) 24-bp imperfect IRRs identified in the flanking insertion sequences, ISPpu12L and ISPpu12R; (ii) identification of an ORF in IS- 

There are some additional features of DEH worth noting. First, the element cloned from the TOL plasmid pWW0 and described by Thomas et al. (44, 45) was a hybrid, presumably formed by recombination between ISPpu12R and ISPpu12 from pWW0 (56) during transposition of DEH from the original donor (i.e., the chromosome of strain PP3) to pWW0. The presence of an element essentially identical to ISPpu12 in pWW0 was a remarkable coincidence, explaining the hot spot for DEH in this region (44). The pWW0 sequence (13) and the further characterization of ISPpu12 of pWW0 described by Williams et al. (56) in the accompanying paper showed that the minor sequence differences between ISPpu12 in PP3 and pWW0 were trivial in functional terms. Thus, it may be assumed that the activities of these two elements are the same and that the same results would have been obtained from the transposition experiments described in this paper even if they had been carried out with DEH cloned directly from PP3 instead of with the hybrid ISPpu12 element produced by recombination between ISPpu12R from PP3 and ISPpu12 resident in pWW0. In addition, since Williams et al. (56) were able to conclude that only TnpA was absolutely required for transposition of ISPpu12 in pWW0, it is reasonable to suggest that the same would hold true for ISPpu12 in PP3.

Second, the tnpA-IRL end of ISPpu12 was found to be identical to a putative insertion sequence designated ISI396 in plasmid R471a originally described by Kulaeva et al. (28). Those authors did not demonstrate transposition of ISI396 and our further analysis of the region upstream of its tnpA gene (2,150 bp available from the sequence reported by Kulaeva et al. [28], including an ORF identified as ispA) showed 100% sequence identity with ISPpu12. This strongly suggests that the identification of ISI396 as an insertion sequence and the IRRs proposed by Kulaeva et al. (28) were incorrect and that the region of the plasmid R471a sequence annotated as ISI396 is partially a copy of ISPpu12.

Third, the fact that DEH transposed to sites in pWW0 and other plasmid targets (e.g., RP4-5) that did not contain ISPpu12-like elements (44) indicated that ISPpu12 copies in DEH were actively involved in transposition. However, the structure of DEH elucidated here provided no obvious explanation as to the mechanism by which transposition of DEH into pWW0 and RP4-5 plasmid targets gave rise to insertions varying from 6 to 13 bp in size (44). DEH products of different sizes may result from the arrangements of ISPpu12 copies in the PP3 genome and/or complexities in the ISPpu12-mediated transposition mechanism. Southern hybridization experiments (L. L. Lee and A. J. Weightman, unpublished results) suggested that the PP3 genome contains only one copy of DEH (i.e., single copies of dehI, dehRI, ISPpu12L, and ISPpu12R) as delineated in Fig. 1, but a part(s) of ISPpu12 was present outside the region of DEH (47). To date ca. 10 kb of the region downstream from tnpA in ISPpu12L on the PP3 chromosome has been sequenced, and we have just started to sequence from ISPpu12R in the opposite direction (L. L. Lee, R. E. Dodds, K. E. Hill, and A. J. Weightman, unpublished results), but no ISPpu12 sequences or other insertion sequence elements have so far been located.

Fourth, the frequency of transposition of DEH-ISPpu12 and the effects of transposition on the target were extreme. The use of a suicide donation system prevented the calculation of transposition frequencies per plasmid transfer, but the fact that after a 6-h filter mating ca. 5% of all potential recipients had received a copy of DEH and/or the inverse transposition product indicated that the transposition frequency was very high.
indeed. Also, since ISPpu12 was able to transpose independently but there was no selection to detect such events, the proportion of recipients that received ISPpu12 may have been even higher than 8%. By comparison, van der Meer et al. (52) used the pSUP202 donation system and reported that the catabolic transposon Tn5280 was recovered in transconjugants at 1/10,000 the frequency of ISPpu12. The observed ratios of true-to inverse-transposition products in recipients suggested that TnpA showed no preference for IRR or IRL. With pAWT34 as a donor, a preference for IRR would have favored DEH (true) transposition, while a preference for IRL would have favored vector (inverse) transposition. This differs from the case for other composite transposons, such as the well-characterized Tn5 and Tn10, the transposases of which show preferences for the outer IRs of IS50 and IS10, respectively (4, 27).

Also, although independent transposition of insertion sequence elements contained in composite transposons is rarely investigated, the fact that independent transposition of ISPpu12 was observed at high frequencies without selection makes it quite unusual.

The ability of DEH-ISPpu12 to produce multiple insertions is noteworthy and was also shown to be the case with transposition of ISPpu12::KmR derivatives reported by Williams et al. (56). This might provide a potential explanation for the gross genomic rearrangements observed in recipients (Fig. 5), in that recombination between multiple copies of DEH-ISPpu12 distributed around the recipients' genomes could have resulted in extensive deletions and/or insertions. The results from the present study did not allow us to determine whether multiple transpositions of DEH-ISPpu12 took place from the same pAWT34 delivery or whether sequential transposition of the element followed insertion of a single copy into the target genome. Also, further work will be required to determine why multiple insertions of the composite transposon were only associated with true transposition and not with inverse transposition (cf. Fig. 3 and 4).

Mutagenesis associated with DEH-ISPpu12 transposition was evident. Indeed, it would seem quite possible that a proportion of recipients may have sustained lethal mutations and would not have been detected, as a result of the element's high transposition activity, promiscuity, and potential to cause multiple gene knockouts and target rearrangements. This raises interesting questions regarding the control of DEH-ISPpu12 activity in strain PP3. The transposition of DEH-ISPpu12 exogenously introduced into strain PP3 produced some of the most intriguing results, in that it was clearly associated with high-frequency silencing of dehI and dehII (Table 1), leading to the observed 2MCPA-negative and DCA-resistant phenotypes. Previously, dehalogenase silencing was observed phenotypically in response to environmental stress, for example, direct selection for resistance to the toxic dehalogenase substrates such as DCA (41, 54). The results in this paper implicate DEH-ISPpu12 in dehalogenase gene silencing and have provided a firm basis for our current investigations into the silencing mechanism. In particular, it will be interesting to discover whether the portable promoter, Pout, in ISPpu12 has any role in dehalogenase gene activation, as seen in the activation of phenol-degradative genes by an outward-directed promoter in IS1411, which was also assigned to the ISL3 family (24).

In addition to ISPpu12 copies in pWW0 (56) and R471a (28), several other ISPpu12-like elements have been identified. Our group (K. E. Hill and A. J. Weightman, submitted for publication) recently reported the isolation of a group of related IncP catabolic plasmids carrying dehalogenase genes, most of which also contained a region that hybridized with ISPpu12 probes. Tsai et al. (48) described the cloning and characterization of a 6-kb DNA segment containing an ohb operon from P. aeruginosa strain 142, encoding enzymes catalyzing oxygenolytic ortho dehalogenation of halobenzoates. They identified an “IS1396-like sequence” just upstream from ohbB, and our analysis of their data showed that this sequence contained the IRL of ISPpu12 7 bp upstream from the ohbB terminator. Only part of this IS1396-like sequence, containing the TnpA ORF and part of the LspA ORF, is available, but again it seems likely that part or all of ISPpu12 is linked to this ohb operon. Bolognese et al. (5) reported that a 3-kb insertion sequence designated ISP1, also related to the ISL3 family, mediated activation and inactivation of aromatic hydrocarbon catabolic pathway genes in P. stutzeri. This element is clearly related to ISPpu12 in that it contained the same tnpA gene (99% nucleotide sequence identity) and was bordered by almost identical IRs; however, ISP1 and ISPpu12 differed with respect to their other ORFs. The involvement of this ISP1 in xyl gene activation and inactivation is of considerable interest given the presence of ISPpu12 in the archetypal TOL plasmid pWW0 and the latter’s association with xylE activation (18, 56).

Thus, there is emerging evidence regarding the distribution of ISPpu12-like elements that also points towards their general involvement in transposition and catabolic gene switching. Recent searches of currently available sequence data from bacterial genome projects, including those for P. putida strains KT2440 and PRS1, have identified only one copy of ISPpu12 (3,367 identical nucleotides of 3,372) and several shorter sequences almost identical to regions of ISPpu12 in Burkholderia fungorum strain LB400 (L. L. Lee and A. J. Weightman, unpublished results). Significant matches were also found between the ORFs in ISPpu12 and several prokaryotic genome sequences, mainly from species of Proteobacteria. However, DEH remains the only ISPpu12-based transposon identified to date.

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