Complete Nucleotide Sequence of TOL Plasmid pDK1 Provides Evidence for Evolutionary History of IncP-7 Catabolic Plasmids^{∇†}

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To understand the mechanisms for structural diversification of *Pseudomonas*-derived toluene-catabolic (TOL) plasmids, the complete sequence of a self-transmissible plasmid pDK1 with a size of 128,921 bp from *Pseudomonas putida* HS1 was determined. Comparative analysis revealed that (i) pDK1 consisted of a 75.6-kb IncP-7 plasmid backbone and 53.2-kb accessory gene segments that were bounded by transposon-associated regions, (ii) the genes for conjugative transfer of pDK1 were highly similar to those of MOB_H group of mobilizable plasmids, and (iii) the toluene-catabolic (*xyl*) gene clusters of pDK1 were derived through homologous recombination, transposition, and site-specific recombination from the *xyl* gene clusters homologous to another TOL plasmid, pWW53. The minireplicons of pDK1 and its related IncP-7 plasmids, pWW53 and pCAR1, that contain replication and partition genes were maintained in all of six *Pseudomonas* strains tested, but not in alpha- or betaproteobacterial strains. The recipient host range of conjugative transfer of pDK1 was, however, limited to two *Pseudomonas* strains. These results indicate that IncP-7 plasmids are essentially narrow-host-range and self-transmissible plasmids that encode MOB_H group-related transfer functions and that the host range of IncP-7-specified conjugative transfer was, unlike the situation in other well-known plasmids, narrower than that of its replication.

Bacterial genes for the utilization of recalcitrant environmental pollutants such as herbicides, pesticides, and petroleum and other industrial waste compounds are often found on plasmids and chromosomally specified integrative and conjugative elements (ICEs) (57). Although the origins of such catabolic genes still remain unknown, it seems most likely that, once established, the catabolic gene modules spread between plasmids and chromosomes through intracellular movements of insertion sequence (IS)-flanked composite transposons (68) and class II (Tn3-related) transposons (61, 63) and intercellular conjugative transfers of plasmids and ICEs (36, 65). The genes associated with the degradation of man-made xenobiotic compounds (e.g., atrazine, 2,4-dichlorophenoxyacetate, and haloacetates) have predominantly been found on broad-host-range and incompatibility group P-1 (IncP-1) plasmids, whereas the genes responsible for the degradation of natural aromatic hydrocarbons (e.g., phenol, naphthalene, and toluene/xylenes) via the *meta*-cleavage catabolic pathways are mainly located on IncP-2, IncP-7, and IncP-9 plasmids, which have been found exclusively in *Pseudomonas* species (38).

Studies of the archetypal 119-kb and IncP-9 toluene/xylene-catabolic (TOL) plasmid pWW0 from *Pseudomonas putida*

mt-2 have greatly contributed to the detailed clarification of genetic and biochemical mechanisms for the aerobic degradation of toluene/xylenes (72). The pWW0-specified *xyl* genes are organized as four transcriptional units within the class II transposons, Tn4651 and Tn4653 (62): (i) the upper pathway operon (*xylXYZLTEGFJQKIH*) for the conversion of toluene and xylenes to benzoate and its methyl derivatives, respectively; (ii) the *meta* pathway operon (*xylXYZLTEGFJQKIH*) for the subsequent conversion to tricarboxylic acid (TCA) cycle intermediates; (iii) and the two transcriptional regulator genes, *xylS* and *xylR* (42). After the discovery of pWW0, various TOL plasmids carrying the *xyl* gene clusters very similar to those on pWW0 were discovered in soil bacteria from geographically distant areas around the world (5, 22, 23, 26, 50, 71). Such TOL plasmids differ in sizes, incompatibilities, and the numbers and relative orientations of *xyl* gene clusters, implying the plausible movement and other rearrangements of *xyl* genes between and within plasmids (5, 70). The rearrangement of catabolic gene clusters is considered to be an important step for the host cell to improve its performance of the preexisting catabolic function(s) (8, 43, 75). Our major aim is to clarify the underlying mechanisms of the recombinations which generate diverse catabolic gene clusters. Comparison of the pWW0 sequence and its related catabolic genes from different *Pseudomonas* strains suggested that the recombination between IS copies on two different replicons led to the establishment of the pWW0-type configuration of the *xyl* gene cluster (70). Our recent studies of an IncP-7 TOL plasmid, pWW53, which possesses two *meta* operons (*meta* 1 and *meta* 2) and three *xylS* genes (two are functional but one is truncated) revealed that (i) pWW53 acquired all of its *xyl* gene clusters through transposition of class

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II transposon Tn4660 and (ii) the site-specific recombination mediated by a transposon-encoded resolvase gave rise to an additional transposon (Tn4656) that carried part of the *xyl* gene cluster on pWW53 (60, 73). These studies implicated transposition-related genes in the rearrangements and dissemination of *xyl* gene clusters.

Another TOL plasmid pDK1 was discovered in a 3-methylbenzoate-utilizing *P. putida* strain, HS1, that was isolated in Minnesota (26). The restriction maps of regions covering the *xyl* genes and other available nucleotide sequences of pDK1 showed remarkable similarity to those of pWW53 (1–3, 41, 51, 67). However, the relative orientation of the *xyl* upper and *meta* operons on pDK1 differs from that of the upper and *meta* 2 operons in pWW53 (see Fig. S1 in the supplemental material). Furthermore, one of the two *meta* operons (*meta* 1) of pWW53 is absent from pDK1 (1), and pDK1 is self-transmissible, in contrast to pWW53 (26). To elucidate the evolutionary relationship between pWW53 and pDK1, we have determined the complete sequence of pDK1. Comparative analysis of the two plasmids strongly suggests their diversification from an ancestral IncP-7 TOL plasmid through both homology-dependent and site-specific recombination events. We also investigated in this study the host range of IncP-7 plasmids.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown at 37°C in Luria-Bertani (LB) broth (48), and *Pseudomonas*, *Sphingomonas*, and *Burkholderia* strains were grown at 30°C in LB broth diluted 1/3 (1/3-LB broth) (74). Solid media were prepared by the addition of 1.5% agar. Antibiotics were added at final concentrations of 10 µg/ml for gentamicin (Gm), 20 µg/ml for tetracycline (Tc), 50 µg/ml for kanamycin (Km), 100 µg/ml for rifampin (Rif), and 20 µg/ml for nalidixic acid (Nal). M9 minimal agar (48) supplemented with 5 mM 3-methylbenzoate (3MB) as a sole source of carbon and energy was used to select the *Pseudomonas* strains carrying the pDK1 derivatives with an intact *xyl meta* pathway operon.

The details of construction of antibiotic-resistant mutants of *Pseudomonas* strains are described in the supplemental material. Curing of pDK1 from *P. putida* HS1 generated HS1C. For this purpose, HS1 was electroporated with a Km-resistant (Km^r) and unstable IncP-7 mini-pCAR1 plasmid, pUCARori004Δpar (54), to obtain the Km^r transformants. The resulting transformant lacking the catabolic activity of 3MB was next cultivated overnight in LB broth without any antibiotics so as to facilitate the spontaneous loss of the unstable pUCARori004Δpar replicon. One of the Km-sensitive derivatives without any plasmids was HS1C.

Conjugation experiments. Filter matings on LB agar plates used for the construction of plasmids and liquid matings in 1/3-LB broth for the transfer of pDK1 derivatives were performed at 30°C for approximately 12 h (69). In liquid matings, approximately a 1:5 ratio of donor and recipient cells in log phase was used.

Standard DNA manipulation and construction of plasmids. CaCl₂-induced competent cells of *E. coli* were subjected to heat shock transformation and used for DNA cloning (48). Electrocompetent cells of *Pseudomonas* strains were prepared according to the protocol by Choi et al. (7). PCR was carried out using KOD-plus high-fidelity DNA polymerase (Toyobo, Osaka, Japan). Extraction of DNA fragments from agarose gel was performed using a QiaEX II bead kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Standard alkaline lysis with phenol-chloroform purification (48) and a LaboPass minikit (Hokkaido System Sciences, Sapporo, Japan) were used for the large- and small-scale preparations, respectively, of plasmids from *E. coli*. The DNA sample prepared by the large-scale preparation was used for shotgun sequencing analysis.

pDK1K, a derivative of pDK1 with an insert of the Km^r gene in the *xylU* gene, and a cointegrate of pDK1K and pJP5608 (40), designated pDK1K::pJP, were constructed according to the procedures described in the supplemental material. A mini-pDK1 plasmid, pMM68, was constructed by cloning of the 6,870-bp *repA-oriV-parWASB*-containing HindIII fragment of pDK1K::pJP into the HindIII site of pK18mob (49), while a mini-pCAR1 plasmid, pMM67, was

constructed by cloning the HindIII fragment containing *repA-oriV-parWASB* region from pUCARori004 (54) into the HindIII site of pK18mob.

Sequencing and annotation. The pDK1K::pJP DNA purified from DH5α (*λpir*) by the large-scale preparation was partially digested with Sau3AI, shotgun cloned into the BamHI site of pUC18, and sequenced. The sequencing reaction was carried out using a BigDye terminator kit, version 3 (Applied Biosystems, Foster City, CA), and the sequencing determination was performed with ABI Prism model 310 and 3730 sequencers (Applied Biosystems). The PCR-based gap-closing process was performed using wild-type pDK1 DNA as a template. Annotation and sequence comparison were carried out using GLIMMER 3 (10), BLAST (16), GenomeMatcher (39), and GENETYX-MAC, version 13 (Genetyx, Tokyo). The Web-based analysis software programs Jpred3 (<http://www.compbio.dundee.ac.uk/~www-jpred/>) (9) and SOSUI (<http://bp.nuap.nagoya-u.ac.jp/sosui/>) (20) were used for the prediction of protein secondary structures and the identification of the signal peptide and transmembrane helix, respectively. The putative transcriptional promoter and Rho-independent terminator were predicted using BPROM and FindTerm, respectively (Softberry).

Nucleotide sequence accession number. Nucleotide sequence of pDK1 was submitted under DDBJ/EMBL/GenBank accession number AB434906. In the annotation of the pDK1 genome, a gene name was not given for a protein with ambiguous function, and such a gene was designated *ofn* (*orf* with no name).

RESULTS AND DISCUSSION

Strategy of pDK1 sequencing. The pDK1 DNA samples prepared from the original *P. putida* host strain (HS1) were always contaminated with a large amount of chromosomal DNA. To overcome this, we attempted to prepare high-purity plasmid DNA from the *E. coli* cells. For this purpose, a pDK1 derivative, pDK1K::pJP, which carried the RP4-derived *oriT* (*oriT*_{RP4}) and R6K-derived *oriV* regions, was first constructed in the HS1 background (see the supplemental material). The former region was expected to allow the conjugative transfer of pDK1K::pJP to *E. coli* in the presence of transfer genes of RP4, and the latter region allows the autonomous replication of pDK1K::pJP in the *E. coli* strain with the *pir* gene that encodes the R6K-specific replication protein. The triparental mating led to the successful conjugative transfer and autonomous replication of pDK1K::pJP in *E. coli* DH5α (*λpir*). Shotgun sequencing of pDK1K::pJP prepared from DH5α (*λpir*) generated five contigs with five gaps. The gap closing was performed by PCR-based walking approaches to obtain the complete sequence of pDK1K::pJP.

Overview of pDK1 genome. *In silico* elimination of the artificially integrated DNA regions (Km^r gene and pJP5608) from the pDK1K::pJP sequence indicated that pDK1 was 128,921 bp in size (accession number AB434906) with an average G+C content of 55.9%. Although five sequences of the pDK1 *xyl* gene clusters (25,644 bp in total) had already been deposited by several other groups (GenBank accession numbers AF134348, L02642, AF019635, M65205, and L02358), our subsequent analysis employed only the pDK1 sequence determined in this study. pDK1 carried 114 probable coding sequences (CDSs) (see Table S1 in the supplemental material). Among them, 22 were predicted to be involved in catabolism and transport of toluene/xylenes (*xyl* and *ben*), 6 are involved in replication and partition (*rep*, *par*, and *tus*), 19 are involved in conjugative DNA transfer (*trh*, *tra*, *ofn64*, *ofn65*, and *ofn72*), 7 are involved in transposition and site-specific recombination (*tnp* and *ist*), 8 are involved in DNA-associated functions (*ssb*, *recT*, *ofn41*, *ofn61*, *ofn80*, *ofn81*, *ofn83*, and *ofn84*), and 51 are involved in other known or unknown functions. Seventeen CDSs were predicted to code for membrane-associated proteins and were mostly located within the conjugative DNA

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s) ^a	Reference or source
Strains		
<i>E. coli</i>		
S17-1	<i>recA pro thi hsdR</i> ; chromosomally integrated RP4-2-Tc::Mu-Km::Tn7; Tp ^r Sm ^r	55
HB101	<i>recA13 hsdS20</i> (r _B ⁻ m _B ⁻) <i>ara-14 proA2 lacI1 galK2 rpsL20 xyl-5 mtl-1 supE44</i>	48
DH5α	λ ⁻ φ80dlacZΔM15 Δ(<i>lacZYA-argF</i>)U169 <i>recA1 endA1 hsdR17</i> (r _K ⁻ m _K ⁺) <i>supE44 thi-1 gyrA relA1</i>	48
DH5α (λpir)	DH5α derivative, λpir lysogen	Laboratory collection
<i>P. putida</i>		
HS1	Soil isolate, carrying pDK1; TOL ⁺ 3MB ⁺	26
HS1C	HS1 derivative cured of pDK1	This study
HS1CR	Spontaneous rifampin-resistant mutant of HS1C; Rif ^r	This study
HS1CG	HS1C::TnMod-OGm; Gm ^r	This study
KT2440	Type strain	ATCC 47054
KT2440G2	KT2440::mini-Tn7-TGm; Gm ^r	This study
F1	Soil isolate	13
F1G	F1::TnMod-OGm; Gm ^r	This study
<i>P. fluorescens</i>		
Pf-5	Type strain	ATCC BAA477
Pf-5S	Spontaneous streptomycin-resistant mutant of Pf-5; Sm ^r	This study
Pf-5G	Pf-5::mini-Tn7-TGm; Gm ^r	This study
<i>P. aeruginosa</i>		
KG2512	<i>P. aeruginosa</i> PAO1 <i>aph</i> ; Km-sensitive strain	N. Gotoh, Kyoto Pharmaceutical University
KG2512G	KG2512::mini-Tn7-TGm; Gm ^r	This study
<i>P. resinovorans</i>		
CA10dm4RG	pCAR1-free derivative of <i>P. resinovorans</i> CA10 with chromosomal copy of TnMod-OGm; Rif ^r Gm ^r	54
<i>S. paucimobilis</i>		
IAM12578G	IAM12578::TnMod-OGm; Gm ^r	32
<i>B. multivorans</i>		
ATCC 17616G	ATCC 17616::TnMod-OGm; Gm ^r	32
Plasmids		
pEX18Tc	pUC replicon carrying <i>sacB</i> ; Mob ⁺ Tc ^r	21
pK18mob	pMB1 replicon; Mob ⁺ Km ^r	49
pJP5608	R6K replicon; Mob ⁺ Tc ^r	40
pUC4K	pUC replicon; Km resistance gene cassette donor; Ap ^r Km ^r	66
pUCARori004ΔparA	pUC19 carrying the <i>repA-oriV-parW-parA-parS-parB</i> region of pCAR1; <i>parA::kan</i> ; Ap ^r Km ^r ; unstable in <i>P. putida</i> due to inactivation of <i>parA</i>	54
pUCARori004	pUC19 carrying the <i>repA-oriV-parW-parA-parS-parB</i> region of pCAR1	54
pRK2013	ColEI replicon, helper plasmid for RK2 <i>oriT</i> -mediated conjugative transfer; Tra ⁺ Km ^r	12
pTNS3	R6K replicon carrying <i>tnsABCD</i> genes of Tn7; Ap ^r	6
pUC18-mini-Tn7T-Gm	pUC replicon carrying mini-Tn7:: <i>aacC1</i> ; Ap ^r Gm ^r	6
pTnMod-OGm	pUC replicon carrying mini-Tn5:: <i>aacC1</i> (= TnMod-OGm); Ap ^r Gm ^r	11
pDK1	IncP-7 replicon; Tra ⁺ TOL ⁺ 3MB ⁺	26
pDK1K	pDK1xylU::kan; Tra ⁺ 3MB ⁺ Km ^r	This study
pDK2	RK2::Tn4663; Tc ^r TOL ⁺	51
pEXxylUKm	pEX18Tc carrying the <i>xylUWC</i> region of pDK1 with the <i>kan</i> gene in <i>xylU</i> ; Mob ⁺ Tc ^r	This study
pJPxylY	pJP5608 carrying the <i>xylYZ</i> region of pDK1; Mob ⁺ Tc ^r	This study
pDK1K::pJP	pDK1K derivative with the pJPxylY insertion into the <i>xylY</i> region; Mob ⁺ 3MB ⁺ Km ^r Tc ^r	This study
pHY118	pK18mob carrying the <i>repA-oriV-parW-parA-parS-parB</i> region of pWW53; Mob ⁺ Km ^r	73
pMM67	pK18mob carrying the <i>repA-oriV-parW-parA-parS-parB</i> region of pCAR1; Mob ⁺ Km ^r	This study
pMM68	pK18mob carrying the <i>repA-oriV-parW-parA-parS-parB</i> region of pDK1; Mob ⁺ Km ^r	This study

^a Abbreviations are as follows: Km, kanamycin; Ap, ampicillin; Tc, tetracycline; Gm, gentamicin; Tra⁺, self-transmissible; Mob⁺, mobilizable; TOL⁺, degradation of toluene/xylenes; 3MB⁺, degradation of 3-methylbenzoate.

transfer region. Most of the pDK1-specified gene products showed high (>83%) identities to those of published IncP-7 plasmids: the self-transmissible and carbazole/dioxin-catabolic plasmid pCAR1 (31), the nontransmissible TOL plasmid pWW53 (73), and the nontransmissible naphthalene-catabolic plasmid pND6-1 (30) (see Table S1).

The pairwise BLASTn analysis revealed that two regions of pDK1, totaling 49.3 kb (the 9.1-kb region from coordinates 1 to

9110 and the 40.2-kb region from 80702 to 120926) showed high (>98%) identities to the DNA regions on pWW53 (Fig. 1). The first carried the genes for replication and partition and also shared high degrees of sequence identity with the corresponding regions on pCAR1 and pND6-1. The second, 40.2-kb, region carried part (here referred to as Tn4660Δ) of the toluene-catabolic transposon Tn4660 from pWW53 (Fig. 1 and 2). In the pWW53 genome, Tn4660 is inserted within a puta-

FIG. 1. Circular map of pDK1. CDSs are shown as boxes inside or outside the circle with gene names or *ofn* numbers. Color indicates proposed functions of gene products: green, replication, partition, and DNA-processing; blue, transposition and site-specific recombination; purple, conjugative DNA transfer; red, degradation of toluene/xylenes; yellow, other known or unknown functions; and gray, gene remnants. Transposon-derived segments are represented by blue arcs. Terminal IRs and *res* regions of class II transposons are indicated as filled triangles and open circles, respectively. The segments highly homologous to those in pWW53, pND6-1, and pCAR1 (revealed by pairwise BLASTn analysis) are indicated by black, gray, and white arcs, respectively.

tive nuclease gene equivalent to ORF59 of pCAR1 and is flanked by direct repeats (Fig. 2) (73). Furthermore, one extremity of Tn4660 is occupied by a nested class II transposon Tn4659. The Tn4659-proximal end of Tn4660 and the truncated ORF59-like gene on pWW53 were also present in the pDK1 genome, suggesting that pWW53 and pDK1 originated from a common ancestral IncP-7 plasmid that had received a Tn4660-like ancestral transposon into the same position (Fig. 2). The other extremity of Tn4660, which contains the *xyl meta* 1 region, was absent from pDK1. Instead, part of a class II transposon, TnAtcArs (64), was present next to the *xyl* upper operon in pDK1. The complex structure of the Tn4660Δ region in pDK1 suggests its structural rearrangement(s) after insertion of the Tn4660-like transposon into the ancestral plasmid of pDK1 (see below).

In contrast to the sequence similarity in the two regions described above, pDK1 differed from pWW53 in the presence of a 63.8-kb region (from inverted repeat 2 [IR2] to *ofn*84, coordinates 16311 to 80150) that carries the genes for conjugative DNA transfer (Fig. 1; see also Table S1 in the supplemental material). The gene organization in this region is the same as that of pCAR1 except for insertion/deletion polymor-

phisms at four sites (Fig. 2). The transfer-related gene products of pDK1 show high (83 to 99%) identities to the corresponding products of pCAR1 but show low (<40%) identities to those of the other reported plasmids. The conservation of the regions for DNA transfer, as well as for replication and partition, between pDK1 and pCAR1 strongly suggests (i) that IncP-7 plasmids originally had a common self-transmissible backbone of at least 75 kb (Fig. 2) and (ii) that nontransmissible IncP-7 plasmids such as pWW53 and pND6-1 have lost their DNA transfer-related genes during their evolution.

Features of replication/partition function region. The 15.5 kb of pDK1 from coordinates 122283 to 8760 contain genes and gene remnants associated with replication (*repA*, *repB*, and *tus*), partition (*parW*, *parA*, and *parB*), and transcriptional regulation (*hns*) (Fig. 3). The gene synteny in this region was conserved in three other IncP-7 plasmids, pDK1, pWW53, and pCAR1, but not in pND6-1; the previously intact *hns* and *tus* gene regions in the ancestral form of pND6-1 must have been replaced by large gene clusters for the catabolism of naphthalene (Fig. 2). The genes necessary for the stable maintenance of pCAR1 have been suggested to be *repA*, *parW*, *parA*, and *parB* (54). The replication initiator protein (RepA) of pDK1

FIG. 2. Schematic representation of insertions and deletions in pDK1, pWW53, pCAR1, and pND6-1. A proposed IncP-7 backbone is represented by a gray bar with landmark genes. pDK1- and pWW53-specific inserts are shown above the backbone, while pCAR1- and pND6-1-specific inserts are shown below the backbone. Genes for the proteins with ambiguous or hypothetical functions in pDK1, pWW53, and pCAR1 are designated *ofn*, *ww*, and *ORF*, respectively. Filled arrowheads indicate terminal IRs of class II transposons. The replication/partition/regulation region (the 14-kb segment from *tus* to *hns*) and conjugative DNA transfer region (the 45-kb segment from *trhN* to *ofn74*) are indicated.

was identical to the RepA proteins of pWW53, pND6-1, and the partially sequenced TOL plasmid pL6.5 (59) and 98.9% identical to the RepA of pCAR1. The RepA proteins of these IncP-7 plasmids were predicted to show structural similarity to replication initiation proteins encoded by iteron-containing plasmids, such as RepA of pPS10 (GenBank accession number CAA41700) and RepE of F plasmid (BAA97915). The copy numbers and relative positions of repeat sequences in the *oriV* regions are the same in pDK1, pCAR1, pWW53, pND6-1, and pL6.5.

Most plasmid partition systems so far characterized are comprised of three components: (i) a centromere, (ii) a DNA-binding protein that specifically recognizes the centromere, and (iii) a partition ATPase that moves the complex of centromere and the binding protein (14). These components correspond to *sopC*, SopB, and SopA, respectively, in the F-plasmid (SopA_F and SopB_F) partition system (33). The ParA protein of pDK1 (ParA_{pDK1}) had an ATP-binding motif typical of a partition ATPase and showed structural similarity to SopA, while the ParB protein (ParB_{pDK1}) showed structural

FIG. 3. Gene organization in replication/partition region of the IncP-7 plasmid. Depicted is the pDK1 segment from coordinates 22283 to 8760. Coding sequences are represented by pentagons. Arrows inserted between *parA* and *parB* indicate the 17-bp palindromic motifs. Gene products required for stable maintenance of the pCAR1 replicon in *P. putida* (54) are indicated in gray. The *repB* and *hns* genes in pDK1 (hatched pentagons) are inactivated by IS1162 insertion and nonsense mutations, respectively (see text for details). The identities of gene products of other IncP-7 plasmids with those of pDK1 are shown below the gene names. To calculate the identities for RepB and H-NS, their respective wild-type proteins of pDK1 are predicted by the *in silico* removal of mutations (the insertion mutation of IS1162 in *repB* and the nonsense mutation at the 38th codon in *hns* in pDK1). NA, not available due to lack of gene homologs in pND6-1. Putative promoters and Rho-independent terminators are indicated by flags and pins, respectively. Two HindIII sites used for construction of mini-IncP-7 replicons, pMM67 (mini-pCAR1), pMM68 (mini-pDK1), and pHY118 (mini-pWW53) (73), are depicted.

similarity to SopB. The ParW proteins encoded by all the sequenced IncP-7 plasmids possessed transmembrane helix motifs at the N-terminal portions, but their detailed function(s) still remains unclear. The ParW, ParA, and ParB proteins of pDK1 were 98 to 100% identical to the respective proteins of pWW53, pDN6-1, and pL6.5 but exhibited relatively low identities to the respective proteins of pCAR1, with 94% identity for ParW, 82% for ParA, and 71% for ParB (Fig. 3). This suggests that pCAR1 is phylogenetically distant from the four other IncP-7 plasmids. General similarity of ParA_{pDK1} and ParB_{pDK1} to SopA_F and SopB_F, respectively, implied the presence of a *sopC*-like region in pDK1. The *sopC* region contains 16-bp palindromic sequence repeats where SopB binds (33). pDK1 and other IncP-7 plasmids had, between their *parA* and *parB* genes, the conserved 17-bp palindrome sequence, 5'-TGGTGCTCGAGCACCA-3' (g, symmetric axis), the copy numbers of which were different among the plasmids: two in pDK1, three in pCAR1, and four in pWW53, pND6-1, and pL6.5 (Fig. 3). This 17-bp motif was not found in the rest of the pDK1 genome. We hypothesized that this 17-bp motif region functions as the centromere designated *parS*. The purified ParB protein of pDK1 was, indeed, able to specifically bind to the *parS* region (our unpublished data).

Features of DNA transfer-associated genes. Conjugative systems in many plasmids from Gram-negative bacteria are comprised of three components: the mating-pair formation (Mpf) apparatus which spans inner and outer membranes and is responsible for the synthesis of the conjugative pilus; the relaxosome, which is a complex of proteins that process the DNA at the origin of transfer (*oriT*), and the coupling protein which connects mating-pair formation apparatus and relaxosome (28). Mobilizable plasmids (MOBs) have recently been classified into six families (MOB_F, MOB_H, MOB_O, MOB_C, MOB_P, and MOB_V) on the basis of the phylogeny of relaxase proteins in the relaxosome components (15). The probable relaxase protein (TraI) of pDK1 (TraI_{pDK1}) showed 96% identity to that of pCAR1, and both TraI relaxases more resembled, in their predicted secondary structures, the relaxase from R27 (a representative of MOB_H group) than the relaxases from other MOB groups (data not shown). TraI_{pDK1} possessed both the three-H motif (HQ-X₂-PASE-X-HHH-X₃-GG-X₃-H-X-L) and the HD hydrolase motif (HD-X-DK), which are uniquely conserved in the MOB_H relaxases (15). Other plausible relaxosome components of pDK1 were Ofn61 and Ofn65. Ofn61 was 26 and 28% identical to the TraO primase of plasmid pIPO2 (MOB_P group) (58) and Orf220 of Rts1, an IncT plasmid, respectively, while Ofn65, exhibiting 44% identity to R0204 of R27 (R0204_{R27}), was a conserved hypothetical protein with a Zn-binding primase-helicase domain (Pfam accession number 8273).

The coupling protein of pDK1, designated TraG, contained an ATPase (Walker A and B) motif and showed 99, 38, and 27% identities, respectively, to the pCAR1, Rts1, and R27 homologs. The Mpf components, which probably constituted the F-plasmid-type retractile pilus, were also shared by pDK1, pCAR1, Rts1, and R27 (see Table S2 in the supplemental material). The Mpf components of pDK1 showed 83 to 99% identities to the pCAR1 homologs, 25 to 46% identities to the Rts1 homologues, and 21 to 33% identities to the R27 homologs. Taking into consideration the conservation of Mpf-

related components among the MOB_H plasmids, the annotation of *mpf* genes of R27 was applied to those of IncP-7 plasmids in this paper. Thus, the genes referred to as *trhF*, *traF*, and *traD* in the pCAR1 paper (31) correspond to *trhP*, *trhF*, and *traG*, respectively, of pDK1.

A total of 48 kb of pDK1 (from *trhN* to *ofn74*) was highly conserved in pCAR1 (Fig. 1) except for the following insertion/deletion polymorphisms: (i) the presence in pDK1 of the IS200/IS605 family transposase gene and the *ofn40* region that are absent from pCAR1, (ii) the replacement of the *ofn38* region of pDK1 by the ISPre4 region in pCAR1, and (iii) the interruption of the *ofn53* region of pDK1 by insertion of ISPre3 on pCAR1 (Fig. 2). In spite of these polymorphisms, pDK1 and pCAR1 possessed their self-transmissibility, indicating that the genes essential for conjugative transfer remain intact in both plasmids.

Analysis with the tBLASTx program (16) revealed that homologs of *ofn71*, *ofn72*, *ofn73*, and *ofn74* were also present in Rts1 as a cluster with a few additional CDSs, suggesting evolution of the cluster as a module; *ofn71*, *ofn72*, *ofn73*, and *ofn74* in pDK1 corresponded to *orf246*, *orf248*, *orf250*, and *orf252*, respectively, in Rts1 (*orf246*_{Rts1}, *orf248*_{Rts1}, *orf250*_{Rts1}, and *orf252*_{Rts1}, respectively). The *oriT* site of Rts1 has been experimentally determined to be located between *orf250*_{Rts1} and *orf252*_{Rts1} (34), and the *oriT* site of pDK1 was assumed to be located between *ofn73* and *ofn74* on the basis of conservation of *oriT*-flanking genes (with *orf250* corresponding to *ofn73* and *orf252* corresponding to *ofn74*). Although repeated sequences and possible stem-loop motifs were present in the probable *oriT* regions of IncP-7 plasmids, no significant sequence similarities in the *oriT* regions were detected between the IncP-7 plasmids and other, better-characterized MOB_H group elements such as R27 (29) and SXT (4). Further experimental approaches are required to elucidate the location of the *oriT* site of pDK1.

Transposons and IS elements. pDK1 carries three, apparently intact, class II transposons (Tn4659, Tn4662, and Tn4663) and an insertion sequence IS1162 (Fig. 1 and 2). Tn4662 and Tn4663 were designated in this study according to the criteria proposed by Roberts et al. (45), whereas IS1162 and Tn4659 have previously been reported (56, 73). Tn4663 (40.8 kb) had two Tn21-like 38-bp terminal inverted repeats (IR3 and IR5) at the ends and contained all the *xyl* genes on pDK1. Shaw and Williams (51) reported that an approximately 40-kb segment of pDK1 including the entire *xyl* gene was able to translocate to RP4 to generate pDK2, leading to the suggestion that the 40-kb segment might be an active catabolic transposon. To determine whether this segment corresponded to Tn4663, the borders of the pDK1-derived segment in pDK2 were sequenced. Our analysis of pDK2 revealed that the entire Tn4663 unit was, indeed, inserted downstream of the *tetR* gene (RP4 coordinate 13245; GenBank accession no. NC_001621) with a 5-bp duplication of RP4 sequence.

An intact class II transposon usually encodes the transpositional cointegration system (the transposase [*tnpA* product] and its target sequences, i.e., terminal IRs) as well as the cointegrate resolution system (the resolvase [*tnpR* product] and its target sequence [*res* region]) (25). Tn4663 encoded two resolution systems; one was encoded within the nested Tn4659 region, and the other was encoded in the 0.7-kb *res-tnpR-IR*

FIG. 4. Comparison of pDK1 and pWW53. (A) Similarity in *xyl*-containing regions of pDK1 and pWW53. Results of BLAST analysis are shown. Pentagons indicate coding sequences. The two *xylS* genes (*xylS1* and *xylS3*) and a truncated *xylS* gene (*xylS2*) are represented by filled pentagons. (B) Sequence comparison among Tn21-related *res* regions. Dots indicate nucleotides identical to those in Tn21. Boxed are the three binding sites of TnpR in the Tn21 *res* region (46) and putatively in the *res* regions in other Tn21-related transposons. The center of site I, where the staggered-cut and subsequent strand exchange takes place, is shaded.

cluster, designated *resX-tnpR*_{TnAtcArs}-IR3 (where *tnpR*_{TnAtcArs} is *tnpR* in TnAtcArs), which was located at the Tn4659-distal end. The nucleotide sequence of *resX-tnpR*_{TnAtcArs}-IR3 was 98% identical to that in the corresponding cluster of an arsenate resistance transposon, TnAtcArs, from *Acidithiobacillus caldus* (64). The *tnpR* and *tnpA* genes in TnAtcArs are divergently transcribed and are physically separated by a 10-kb arsenate resistance (*ars*) operon. The similarity of the *resX-tnpR*_{TnAtcArs}-IR3 region to the original TnAtcArs form suggests that *resX-tnpR*_{TnAtcArs}-IR3 is a remnant of an intact TnAtcArs-like transposon that might be formed by deletion of the *ars-tnpA*-covering portion.

Transposon Tn4662 was very closely related to Tn5501-Tn5502 (27) and Tn5503 (18) in terms of gene content and sequence similarity. Tn4662 differed from Tn5503 on Rms149, an IncP-6 *Pseudomonas* plasmid, only in the repeat number of the 7-bp sequence (5'-CCCAGAG-3') in *ofn19*; the repeat number was 16 in Tn4662 and 9 in the corresponding gene of Tn5503. A deletion derivative of Tn4662 is also located on pND6-1 (30). Although IS1162 was present on pDK1 and pCAR1, the positions of IS1162 were different between the two plasmids. The presence of identical or very closely related transposable elements on pDK1, pND6-1, pCAR1, and Rms149 suggests that these plasmids have evolved in phylogenetically related host strains.

Rearrangements of toluene-catabolic gene clusters. pDK1 has been reported to carry an *xyl meta* operon that was functionally homologous to the pWW53 *meta 2* operon (51). The complete sequence of pDK1 revealed that both the *xyl* upper and *meta* operons of pDK1 were more similar to those from

pWW53 than to those from pWW0 and that the *meta* operon of pDK1 was more similar to the *meta 2* operon than to the *meta 1* operon of pWW53 (the identities of gene products are shown in Fig. S2 in supplemental material), confirming the previous report (51). On the basis of sequence similarity between the *xylS* gene of pDK1 (*xylS*_{pDK1}) and two intact *xylS* genes on pWW53 (*xylS1* and *xylS3* [*xylS1*_{pWW53} and *xylS3*_{pWW53}, respectively]), Assinder et al. (1) proposed that *xylS*_{pDK1} could be a hybrid gene of *xylS1*_{pWW53} and *xylS3*_{pWW53} (the 5' portion of *xylS*_{pDK1} from *xylS1*_{pWW53} and the 3' portion from *xylS3*_{pWW53}). They also suggested that the current organization of *xyl* operons in pDK1, wherein the upper operon is located on the opposite strand relative to the *meta* operon, could have originated from an ancestral form, in which both the upper and *meta* operons are located on the same DNA strand, by homology-dependent intramolecular inversion between two copies of *xylS* genes (1). If the *xyl* gene cluster of pDK1 had originated from the pWW53-type *xyl* gene cluster, pDK1 must have lost one *meta* operon as well as one copy of the *xylS* gene. The loss of such a gene cluster can be explained most simply by the following mechanism.

pDK1 and pWW53 share two highly conserved segments. One is the 45.5-kb segment of pDK1 that starts at IR1 (coordinate 9110) and ends at the *xylS* region (coordinate 95348), and the other is the 14.4-kb segment of pDK1 that starts at the *resX* region (coordinate 80871) and ends in the *xylS* region (coordinate 95251) (Fig. 1 and 4A). In pWW53, the 14.4-kb segment starts from *xylS1* (coordinate 57656) and ends in *res2* (coordinate 72064), which is a remnant of Tn21-like transposon and is functional as a target site of the pWW53-encoded

FIG. 5. Proposed model for generation of a pDK1-type *xyl* gene cluster from a pWW53-type cluster: event 1, transposition of TnAtcArs into the pDK1 ancestor; event 2, homology-dependent intracellular inversion between *xylS1* and *xylS3*; event 3, TnpR-mediated site-specific resolution between *res2* and the *res* region of the TnAtcArs-like transposon. The order of events 1 and 2 is unclear. See text for details.

resolvase (Fig. 4A) (73). The BLAST searches against databases revealed the closest similarities of the 45.5- and 14.5-kb regions of pDK1 to those from pWW53 (see Table S1 and Fig. S2 in the supplemental material). These structural features and the sequence similarity between the two plasmids are consistent with the previous hypothesis (1) that the current *xyl* upper operon-containing region of pDK1 originated from the opposite strand of pWW53 by the intramolecular inversion of the segment comprised of *xylS1*, the upper operon, and *xylS3* (Fig. 5).

The biological role of TnpR and the *res* region is to resolve a cointegrate that is formed in the transposition process of class II transposons. TnpR catalyzes the DNA-cleavage and -rejoining reaction between two directly repeated *res* regions such that the DNA segment flanked by two *res* regions is deleted, leaving one *res* region on a replicon (19). Thus, the loss of the *meta* 1 operon and one *xylS* gene can be explained by the TnpR-mediated intramolecular site-specific recombination between the two *res* regions. The *res* regions of class II transposons are generally comprised of three subsites (sites I, II, and III) to each of which the dimer form of TnpR binds, and crossing-over takes place at the center of site I (44). The *resX* region of pDK1 (*resX*_{pDK1}) showed high similarity to the *res2* region of pWW53 (*res2*_{pWW53}) and the *res* region of TnAtcArs (*res*_{TnAtcArs}) (Fig. 4B). Among the 180-bp *res* regions shown in Fig. 4B, *resX*_{pDK1} and *res2*_{pWW53} share the first 41 bp up to the center of the putative site I sequences, while *resX*_{pDK1} and *res*_{TnAtcArs} share the rest of the 180-bp regions except for two mismatches in the site I sequences. This similarity in the three *res* regions is thus consistent with the site-specific recombination event between *res2* and the *res* region in the TnAtcArs-like transposon. It is possible that the transposition of the TnAtcArs-like transposon downstream of conjugative DNA transfer genes happened to give rise to two directly repeated *res* sites, and subsequently the *meta* 1-*xylS* region, part of the

plasmid backbone, and part of the TnAtcArs-like transposon were eliminated from the pDK1 ancestor by the TnpR_{TnAtcArs}-mediated resolution reaction (Fig. 5).

It has been pointed out that the *res* regions of class II transposons can be hot spots of recombination that facilitate rearrangement and shuffling of replicons through intermolecular and intramolecular recombinations (24). Our analyses of two IncP-7 TOL plasmids have revealed two different examples where two related *res* regions present on a plasmid have led to rearrangements. One is the case of pWW53, in which two related *res* regions (*res2* and Tn4658 *res*) with inverse orientations caused the inversion of pWW53, leading to the generation of another class II transposon, Tn4656 (73). The other case is described in this paper: the transient formation of directly repeated *res* regions on a plasmid resulted in the deletion of the segment flanked by the two *res* regions (Fig. 5). Interestingly, the rearrangement of pDK1 also generated a toluene-catabolic transposon, Tn4663, which moved to RP4 to form pDK2 (51). These observations suggest that the resolution system contributes to the formation of class II transposons with novel genetic contexts.

The host ranges of IncP-7 plasmids. The complete sequence determination of pDK1 has provided insights for genetic organization of the ancestral IncP-7 plasmid (Fig. 2). However, available information for the IncP-7 group was still limited with regard to basic plasmid functions and the range of its potential hosts (47, 52, 53). To obtain the consensus information on host ranges of IncP-7 plasmids, minireplicons of three IncP-7 plasmids (pHY118 for pWW53, pMM67 for pCAR1, and pMM68 for pDK1) that carried the *repA-oriV-parWASB* region (Fig. 3) were introduced from *E. coli* S17-1 into five plasmid-free *Pseudomonas* strains (i.e., the derivatives of *Pseudomonas resinovorans* CA10 [designated CA10dm4G], *P. putida* F1 [F1G], *P. putida* KT2440 [KT2440G2], *Pseudomonas aeruginosa* PAO1 [KG2512G], and *P. fluorescens* Pf-5 [Pf-5G])

as well as the pDK1-free derivative of HS1 (HS1CG) through the RP4-specified conjugation machinery (the *oriT*_{RP4} site on the three minireplicons and all of the *trans*-acting transfer-related genes from the S17-1 chromosome). We additionally included *Sphingomonas paucimobilis* IAM12578G (an alpha-proteobacterial strain) and *Burkholderia multivorans* ATCC 17616G (a betaproteobacterial strain) as recipients. The three minireplicons were transferred into all six *Pseudomonas* strains at frequencies of 10^{-5} to 10^{-2} transconjugants per donor cell but not to the *S. paucimobilis* or *B. multivorans* strain. These results indicate that IncP-7 plasmids are able to replicate at least in the *Pseudomonas* strains examined but not in alpha- or betaproteobacteria.

Conjugative transfer of pDK1 was next investigated using pDK1K, a pDK1 derivative having a Km^r gene in *xylU*. Among the six plasmid-free *Pseudomonas* strains that were employed as the recipient in the pMM68 transfer (see above), pDK1K was transferred from *P. putida* HS1CR to *P. putida* HS1CG and *Pseudomonas fluorescens* Pf-5G at frequencies of 4.4×10^{-4} and 2.5×10^{-3} , respectively, per donor cell. When *P. fluorescens* Pf-5S was used as a donor strain, the plasmid was transferred to Pf-5G and HS1CG at frequencies of 3.0×10^{-5} and 2.3×10^{-7} , respectively, per donor cell. No transfer of pDK1K was detected ($<10^{-8}$ per donor cell) to *P. putida* F1G, *P. putida* KT2440G2, *P. resinovorans* CA10dm4G, or *P. aeruginosa* PAO1G. This indicates that pDK1 retains self-transmissibility but with a limited recipient range even in *P. putida* strains. This property contrasted with the replication ability of pMM68 in the six *Pseudomonas* strains. The limited recipient range of conjugative transfer is also associated with pCAR1 (53), and it is therefore most likely that the host range of self-transmissible IncP-7 plasmids is, in general, restricted by the conjugation system rather than the replication system.

Conclusions. The primary purpose of this study was to clarify the evolutionary relationship between the two TOL plasmids pDK1 and pWW53. Analysis of the pDK1 genome revealed the evolutionary history of catabolic gene clusters on this plasmid and the conservation of genes for basic plasmid functions among IncP-7 plasmids. We additionally performed experimental analyses to obtain insights for the host range of the IncP-7 group. Four major findings concerning the nature of IncP-7 plasmids from this study are summarized as follows. First, pDK1 and pWW53 shared 49.3 kb that cover the plasmid backbone region and the catabolic transposon region, and this clearly demonstrated that the two plasmids have originated from an ancestral IncP-7 TOL plasmid. Second, generation of the *xyl* gene cluster on pDK1 was most simply explained by the three sequential recombination events: (i) homology-dependent inversion between two copies of an *xylS* gene, (ii) insertion of a Tn*AtsArs*-like transposon, and (iii) the TnpR_{Tn*AtsArs*}-mediated site-specific deletion between directly repeated *res* regions (Fig. 5). Third, the limited replication host ranges of pDK1 and pCAR1 and the conservation of MOB_H group-related DNA transfer genes in pDK1 and pCAR1 suggest that the IncP-7 plasmids are narrow-host-range and self-transmissible plasmids specialized to *Pseudomonas* strains (52). Lastly, for several *Pseudomonas* strains in which the mini-pDK1 plasmid could replicate, no conjugative transfer of pDK1 was detected. This result contrasted with the paradigm that the host

range of transfer is broader than that of replication in other self-transmissible plasmids (17).

Several TOL plasmids other than pWW53 contain two homologous or higher-similarity *meta* pathway operons (37, 50), and there is a report indicating that the *xyl*-related catabolic genes are located on the chromosomes (35). However, the relationship of these catabolic genes to mobile genetic elements is still unclear. Sequence analyses of such catabolic gene clusters and their flanking regions will provide many clues leading to a better understanding of how functional gene clusters are assembled and disseminate in nature.

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