

## Broad-Host-Range Plasmids from Agricultural Soils Have IncP-1 Backbones with Diverse Accessory Genes<sup>∇</sup>

Diya Sen,<sup>1,2</sup> Geraldine A. Van der Auwera,<sup>3</sup> Linda M. Rogers,<sup>1</sup> Christopher M. Thomas,<sup>4</sup>  
Celeste J. Brown,<sup>1,2</sup> and Eva M. Top<sup>1,2\*</sup>

Department of Biological Sciences, Institute for Bioinformatics and Evolutionary Studies (IBEST),<sup>1</sup> and Bioinformatics and Computational Biology Graduate Program,<sup>2</sup> University of Idaho, Moscow, Idaho 83844-3051; Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115<sup>3</sup>; and College of Life and Environmental Sciences, School of Biosciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, United Kingdom<sup>4</sup>

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**Broad-host-range plasmids are known to spread genes between distinct phylogenetic groups of bacteria. These genes often code for resistances to antibiotics and heavy metals or degradation of pollutants. Although some broad-host-range plasmids have been extensively studied, their evolutionary history and genetic diversity remain largely unknown. The goal of this study was to analyze and compare the genomes of 12 broad-host-range plasmids that were previously isolated from Norwegian soils by exogenous plasmid isolation and that encode mercury resistance. Complete nucleotide sequencing followed by phylogenetic analyses based on the relaxase gene *traI* showed that all the plasmids belong to one of two subgroups ( $\beta$  and  $\epsilon$ ) of the well-studied incompatibility group IncP-1. A diverse array of accessory genes was found to be involved in resistance to antimicrobials (streptomycin, spectinomycin, and sulfonamides), degradation of herbicides (2,4-dichlorophenoxyacetic acid and 2,4-dichlorophenoxypropionic acid), and a putative new catabolic pathway. Intramolecular transposition of insertion sequences followed by deletion was found to contribute to the diversity of some of these plasmids. The previous observation that the insertion sites of a Tn501-related element are identical in four IncP-1 $\beta$  plasmids (pJP4, pB10, R906, and R772) was further extended to three more IncP-1 $\beta$  plasmids (pAKD15, pAKD18, and pAKD29). We proposed a hypothesis for the evolution of these Tn501-bearing IncP-1 $\beta$  plasmids that predicts recent diversification followed by worldwide spread. Our study increases the available collection of complete IncP-1 plasmid genome sequences by 50% and will aid future studies to enhance our understanding of the evolution and function of this important plasmid family.**

Plasmids play a crucial role in the evolution and adaptation of prokaryotes. They typically have mosaic genomes consisting of two distinct regions (34): a backbone region that carries genes essential for plasmid persistence and one or more accessory regions that encode host-beneficial traits. The so-called broad-host-range (BHR) plasmids are of particular importance in rapid bacterial adaptation to changing environments, because they can transfer to and replicate in phylogenetically diverse bacteria at high frequencies and because they often carry host-beneficial traits such as resistances to antibiotics or heavy metals or degradation of organic compounds. In light of the recent emergence of multidrug-resistant pathogens as an acute human health crisis, the study of the diversity and evolutionary history of BHR plasmids has become imperative.

The most promiscuous plasmids of Gram-negative bacteria, able to transfer themselves and replicate in bacteria from two or more classes of *Proteobacteria*, belong to incompatibility groups IncP, IncU, and IncW (7, 11) and the recently defined PromA group (39). Complete sequences of 25 IncP-1, 4 IncW, 4 PromA, and 2 IncU plasmids are currently available in GenBank. With 25 members, the IncP-1 plasmids are the best-

represented and best-studied group of BHR plasmids. This group, initially consisting of only two subgroups,  $\alpha$  and  $\beta$  (16, 33), has been expanded over the last 5 years to include at least three ( $\gamma$ ,  $\delta$ , and  $\epsilon$ ) (2, 13, 41) and possibly five (19; D. Sen, M. L. Bauer, L. M. Rogers, G. A. Van der Auwera, C. J. Brown, and E. M. Top, unpublished data) more subgroups.

In the mid-1990s, a set of plasmids was isolated from Norwegian soils after the soil samples were first treated in the laboratory with mercuric chloride (HgCl<sub>2</sub>) (8, 9). Two approaches to exogenous plasmid isolation were used, biparental and triparental matings. Biparental matings typically aim at capturing plasmids that encode a specific phenotypic trait by mixing the indigenous bacterial community from an environmental sample with a plasmid-free recipient strain, allowing conjugation to occur, and selecting for recipients that acquired the specific trait (here mercury resistance). Triparental matings involve one additional parental population, i.e., a donor strain carrying a nonconjugative, mobilizable plasmid (Tra<sup>-</sup>Mob<sup>+</sup>), and plasmids are captured based solely on their ability to transfer the mobilizable plasmid and themselves into the recipient. In both cases, the transconjugants are then examined for the presence of captured conjugative plasmids. Of the so-called pAKD plasmids (pAKD1 to pAKD34) captured with these methods, 19 had been found to have a broad host range based on their ability to transfer and replicate in different classes of *Proteobacteria*, and all were shown to confer resistance to mercuric chloride. With the exception of one plasmid,

\* Corresponding author. Mailing address: Department of Biological Sciences, University of Idaho, 258 Life Sciences South, Moscow, ID 83844-3051. Phone: (208) 885-5015. Fax: (208) 885-7905. E-mail: evatop@uidaho.edu.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid name	Reference or source
<i>E. coli</i> CV601::gfp.....	14
<i>Cupriavidus pinatubonensis</i> JMP228n.....	36
<i>E. coli</i> EC100.....	Invitrogen, Carlsbad, CA
pEMT1::Km.....	36
pHSG399.....	32

which hybridized to a probe for the IncP-1 $\beta$  subgroup, lack of hybridization of these plasmids with DNA probes known to detect specific incompatibility groups suggested that they belonged to new groups of BHR plasmids (8, 9).

To increase our understanding of the genetic diversity of BHR plasmids, we determined the complete nucleotide sequences of 13 of these 19 BHR plasmids from three Norwegian soils. Based on genomic comparisons and phylogenetic analyses, we found that in contrast to the earlier hybridization results, all plasmids belong to three subgroups of the IncP-1 group ( $\beta$ ,  $\delta$ , and  $\epsilon$ ). Since we have already published a detailed analysis of the IncP-1 $\delta$  plasmid pAKD4 (27), this study presents a comparative analysis of the remaining 12 pAKD plasmids and a preliminary characterization of their accessory regions. Since there are so far only 26 completely sequenced IncP-1 plasmids available to the public, this study increases this group by nearly 50%. The data not only reveal new accessory genes but also help to define the interesting molecular events that took place during the evolution of these plasmids.

#### MATERIALS AND METHODS

**Bacterial strains and plasmids used.** Table 1 provides a list of bacterial strains and control plasmids used in this study. The pAKD plasmids were kindly provided by K. Drønen (8, 9).

**Plasmid DNA extractions.** All plasmids were extracted from the strains in which they were received; thus, pAKD4, pAKD17, and pAKD18 were extracted from *Pseudomonas putida* UWC1 and the other plasmids were extracted from *Escherichia coli* HB101. Plasmid DNA to be used for sequence determination was isolated using the plasmid midikit (Qiagen, Valencia, CA) according to the manufacturer's instructions for low-copy-number plasmids. Plasmid DNA extractions for additional analyses were obtained using the alkaline lysis method (23). Standard methods were used for restriction analysis and gel electrophoresis (23). In all cases, EcoRI digestion of the plasmid DNA that was sequenced yielded the same restriction patterns as those observed previously by Drønen et al. (8, 9).

**Sequencing and annotation.** The DNA sequences of all plasmids were determined by shotgun sequencing at the DOE Joint Genome Institute (Walnut Creek, CA). Briefly, after construction of ~3-kb clone libraries, Sanger technology was used to determine the DNA sequences of 384 clones in both directions. These sequences were then assembled using PGA (42), and gaps were closed by primer walking. Automatic annotation was carried out by the J. Craig Venter Institute Annotation Service (<http://www.jcvi.org/cms/research/projects/annotation-service/overview/>) followed by manual annotation by the authors.

**Bioinformatics analyses and software.** GenBank (<http://ncbi.nlm.nih.gov>) was searched for similar sequences using BLAST (1). Blast searches of all hypothetical proteins were carried out against the Interpro database (<http://www.ebi.ac.uk/interpro/>), a database of protein families, domains, regions, repeats, and sites in which identifiable features found in known proteins can be applied to new protein sequences. The alignment figure of the pAKD plasmids was generated using Mauve (6), ClustalW (36), and TRAPPIST (G. Van der Auwera, unpublished data). The TRAPPIST toolbox was used to perform multiple alignments with Mauve, produce refined pairwise alignments between segments using ClustalW based on the Mauve output, and generate the graphical representation shown in Fig. 2. To infer the phylogenetic relationship of the pAKD plasmids with other sequenced IncP-1 plasmids, amino acid sequences of the relaxase protein (TraI) of 26 IncP-1 plasmids were aligned in ClustalX and this alignment was used to guide the alignment of nucleotide sequences in Tralign (22). A maximum likelihood phylogeny based on this alignment was inferred using

RAxML (30). Accession numbers of the IncP-1 plasmids included in the phylogenetic analysis are as follows: pQKH54 (NC\_008055), pEST4011 (NC\_005793), pIJB1 (DQ065837), pSP21 (CP002153), pB5 (CP002151), pB11 (CP002152), RK2 (NC\_001621), pBS228 (NC\_008357), pTB11 (NC\_006352), pKJK5 (NC\_008272), pA81 (NC\_006830), pCNB (NC\_010935), pB4 (NC\_003430), pAOVO02 (NC\_008766), pA1 (NC\_007353), pB10 (NC\_004840), pJP4 (NC\_005912), pBP136 (NC\_008459), pUO1 (NC\_005088), pTP6 (NC\_007680), *Burkholderia cepacia* AMMD plasmid 1 (pAMMD\_1) (NC\_008385), pADP-1 (NC\_004956), pB3 (NC\_006388), R751 (NC\_001735), pB8 (NC\_007502), and pAKD4 (GQ983559).

**Copy number analysis.** SYBR-green-based quantitative PCR (qPCR) was used to compare copy numbers of pAKD16 and pAKD34 as described previously (29). The plasmids were transferred to EC100 (Invitrogen, Carlsbad, CA). The cells were grown overnight in LB supplemented with HgCl<sub>2</sub> (2.5  $\mu$ g/ml) at 37°C and then transferred into fresh LB medium and grown into the exponential phase. The copy number of a 90-bp region within the *trbB* gene was used to calculate plasmid copy number, while chromosomal copy number was calculated for a 167-bp region within the *atpB* gene of the EC100 chromosome, close to *oriC*. The control vector containing a 1:1 ratio of plasmid and chromosomal genes was constructed by cloning *trbB-trbE* and *atpB* into pHSG399 (32). Primers used for qPCR were *trbBF*, 5'-CTCGTTTGAAGTCGCTTGT-3'; *trbBR*, 5'-TTTCTGGATACGACGAGAG-3'; *atpBF*, 5'-GTCGGTCCAGGTCTTCATTT-3'; and *atpBR*, 5'-TGCACACGGTAATCTGGAAT-3'. The ratio of plasmid to chromosomal gene copies was used as a proxy for plasmid copy number.

**Antimicrobial resistance tests.** Plasmid pAKD1 was tested for conferring resistance to streptomycin, spectinomycin, and sulfonamide. Because the original host, *E. coli* HB101, was resistant to streptomycin and spectinomycin, the plasmid was first transferred from *E. coli* HB101 to *E. coli* CV601::gfp (supplied by K. Smalla) using a biparental mating as described previously (27). Selection for transconjugants was performed on LBA medium supplemented with 5  $\mu$ g/ml of mercuric chloride and 150  $\mu$ g/ml of rifampin. The presence of pAKD1 in transconjugants was confirmed after plasmid DNA extraction from randomly selected colonies using the alkaline lysis method and visualization of plasmid DNA on a 0.75% agarose gel (23). *E. coli* CV601::gfp(pAKD1) was tested for resistance to the three antimicrobial compounds. Plates were prepared with Luria-Bertani medium, solidified with 15 g/liter agar, and supplemented with 25, 50, and 100  $\mu$ g/ml of streptomycin; 25, 50, and 100  $\mu$ g/ml of spectinomycin; or 200  $\mu$ g/ml of the sulfonamide sulfathiazole. Overnight cultures of *E. coli* CV601::gfp with and without pAKD1 were streaked onto plates, incubated at 37°C, and observed for colony growth.

**Test for degradation of 2,4-D.** The *tfd* gene-bearing plasmids pAKD25 and pAKD26 were tested for their ability to confer degradation of 2,4-dichlorophenoxyacetic acid (2,4-D) to their bacterial hosts. Biparental matings were used to transfer both plasmids from *E. coli* HB101 to *Cupriavidus pinatubonensis* JMP228n (37) as previously described (27). Transconjugants were selected on LBA plates supplemented with 5  $\mu$ g/ml of mercuric chloride and 100  $\mu$ g/ml of nalidixic acid, and plasmid presence was confirmed as described above. Single colonies of JMP228n(pAKD25) and JMP228n(pAKD26) were isolated and grown overnight in LB. Cells were pelleted and washed with MMO medium (31), and 10<sup>7</sup> cells were inoculated in liquid MMO medium supplemented with 250, 500, and 1000  $\mu$ g/ml of 2,4-D. JMP228n harboring pEMT1::Km (37) was used as a positive control, and plasmid-free JMP228n was used as a negative control. All cultures were grown at 30°C with constant shaking at 200 rpm. Turbidity of cultures was monitored daily for 6 days.

**Test for degradation of 2,4-dichlorophenoxypropionic acid.** Plasmid pAKD34 was transferred to JMP228n by biparental mating as described above. Transconjugants were selected on LBA plates supplemented with 5  $\mu$ g/ml of mercuric chloride and 100  $\mu$ g/ml of nalidixic acid (31), and plasmid presence was confirmed as described above. A single colony of JMP228n(pAKD34) was inoculated in LB overnight, cells were pelleted and washed with MMO medium (31), and 10<sup>7</sup> cells were inoculated in liquid MMO medium supplemented with 250  $\mu$ g/ml of racemic 2,4-dichlorophenoxypropionic acid (also called dichlorprop; Sigma-Aldrich, San Diego, CA). Turbidity of cultures was monitored for 5 days. Triplicate measurements of optical density (at 600 nm) and dichlorprop absorbance (at 280 nm) were recorded on days 2 and 5 for JMP228n(pAKD34) and plasmid-free JMP228n with a spectrophotometer. To measure absorbance, 1 ml of culture was centrifuged and the supernatant was transferred to a UV cuvette. Dilutions of a known concentration of dichlorprop solution in MMO were used as standards to calculate concentrations of dichlorprop in unknown samples.

**Antimicrobial resistance tests.** Plasmid pAKD1 was tested for conferring resistance to streptomycin, spectinomycin, and sulfonamide. Because the original host, *E. coli* HB101, was resistant to streptomycin and spectinomycin, the plasmid was first transferred from *E. coli* HB101 to *E. coli* CV601::gfp (supplied by

TABLE 2. General features of the pAKD plasmids

Plasmid	Soil of origin <sup>a</sup>	Isolation method <sup>b</sup>	Size (bp)	IncP-1 subgroup	Phenotype <sup>c</sup>
pAKD1	StendS	BM	58,246	β	Hg <sup>r</sup> Sp <sup>r</sup> Sm <sup>r</sup> Su <sup>r</sup>
pAKD4 <sup>d</sup>	StendS	BM	56,803	δ	Hg <sup>r</sup>
pAKD14	Ås	BM	48,624	β	Hg <sup>r</sup>
pAKD15	Ås	BM	66,550	β	Hg <sup>r</sup>
pAKD16	Ås	TM	74,971	ε	Hg <sup>r</sup>
pAKD17	Ås	BM	51,755	β	Hg <sup>r</sup>
pAKD18	Ås	BM	67,298	β	Hg <sup>r</sup>
pAKD25	Garpestad	BM	75,067	ε	Hg <sup>r</sup>
pAKD26	Garpestad	BM	82,401	β	Hg <sup>r</sup>
pAKD29	StendS	BM	55,020	β	Hg <sup>r</sup>
pAKD31	Ås	BM	54,140	β	Hg <sup>r</sup>
pAKD33	Ås	BM	57,974	β	Hg <sup>r</sup>
pAKD34	Ås	BM	86,438	ε	Hg <sup>r</sup>

<sup>a</sup> Soil of origin—agricultural fields growing cereal and vegetables in StendS and Garpestad and only cereal in Ås.

<sup>b</sup> Isolation method: BM, biparental mating; TM, triparental mating (8, 9).

<sup>c</sup> Phenotype: phenotype confirmed. Hg<sup>r</sup>, mercury resistance; Sm<sup>r</sup>, streptomycin resistance; Sp<sup>r</sup>, spectinomycin resistance; Su<sup>r</sup>, sulfonamide resistance.

<sup>d</sup> Previously published (27).

K. Smalla) using a biparental mating as described previously (27). Selection for transconjugants was performed on LBA medium supplemented with 5 µg/ml of mercuric chloride and 150 µg/ml of rifampin. The presence of pAKD1 in transconjugants was confirmed after plasmid DNA extraction from randomly selected colonies using the alkaline lysis method and visualization of plasmid DNA on a 0.75% agarose gel (23). *E. coli* CV601::gfp(pAKD1) was tested for resistance to the three antimicrobial compounds. Plates were prepared with Luria-Bertani medium, solidified with 15 g/liter agar, and supplemented with 25, 50, and 100 µg/ml of streptomycin; 25, 50, and 100 µg/ml of spectinomycin; or 200 µg/ml of the sulfonamide sulfathiazole. Overnight cultures of *E. coli* CV601::gfp with and without pAKD1 were streaked onto plates, incubated at 37°C, and observed for colony growth.

**Nucleotide sequence accession numbers.** Complete nucleotide sequences of 12 pAKD plasmids have been submitted to GenBank, and their accession numbers are as follows: pAKD1 (JN106164), pAKD14 (JN106165), pAKD15 (JN106166), pAKD16 (JN106167), pAKD17 (JN106168), pAKD18 (JN106169), pAKD25 (JN106170), pAKD26 (JN106171), pAKD29 (JN106172), pAKD31 (JN106173), pAKD33 (JN106174), and pAKD34 (JN106175).

## RESULTS AND DISCUSSION

**General sequence features.** The complete nucleotide sequences of 12 plasmids previously isolated from three Norwegian soils (8, 9) were determined by Sanger sequencing. All plasmids have regions for replication (*trfA*, *ssb*, *oriV*), conjugative DNA transfer (*tra*, *oriT*), mating-pair formation (*trb*), and stable inheritance and central control (*ctl*). Based on their nucleotide sequences and gene content, they are typical IncP-1 plasmids, with accessory genes situated between *oriV* and *trfA* and/or between the *tra* and *trb* regions. This result was surprising given the previous report of lack of hybridization of all but pAKD1 to IncP-1 plasmid-specific probes (repP<sub>α</sub> and repP<sub>β</sub>), leading to the earlier conclusion that they were novel BHR plasmids (8, 9). General features of these newly sequenced plasmids are listed in Table 2.

**Phylogenetic and comparative genomic analysis of the pAKD plasmid backbones.** To analyze the phylogenetic relationship of the pAKD plasmids with 26 known IncP-1 plasmids, a maximum likelihood tree was inferred using RAxML (30) based on an alignment of the relaxase gene, *traI* (Fig. 1). This gene was chosen as a representative backbone gene since the relaxase protein has recently been proposed as a universal

protein encoded on mobilizable plasmids (10). The tree shows that the 12 newly sequenced pAKD plasmids (shown in bold in Fig. 1) are diverse and belong to two of the five IncP-1 subgroups: IncP-1β and IncP-1ε. This same main tree topology was found when using the replication initiation gene *trfA1* or several other backbone genes (data not shown). These plasmids are clearly distinct from the IncP-1δ plasmid pAKD4, which was isolated from one of the three soils in the same study and described previously (27).

While eight pAKD plasmids (pAKD1, pAKD14, pAKD15, pAKD17, pAKD18, pAKD29, pAKD31, and pAKD33) cluster with the prototype IncP-1β plasmid R751 in the phylogenetic tree, pAKD26 falls within a separate smaller IncP-1β clade that contains multidrug resistance plasmid pB4, catabolic plasmids pCNB-1 and pA81, cryptic plasmid pA1, and so-far-uncharacterized plasmid pAOVO02, which encodes a putative oxidoreductase (Fig. 1). These two clades within the IncP-1β subgroup have recently been designated β-1 and β-2 (18). Moreover, the IncP-1β plasmids from the two subclades are different not only in backbone gene sequence but also in gene content within the *ctl* region (*kfiC* to *kfcA*). The plasmid alignment shown in Fig. 2 clearly indicates the lower sequence and gene content similarity between plasmid pAKD26 and the eight R751-like pAKD plasmids.

The phylogenetic tree also indicates that pAKD16, pAKD25, and pAKD34 belong to the recently proposed

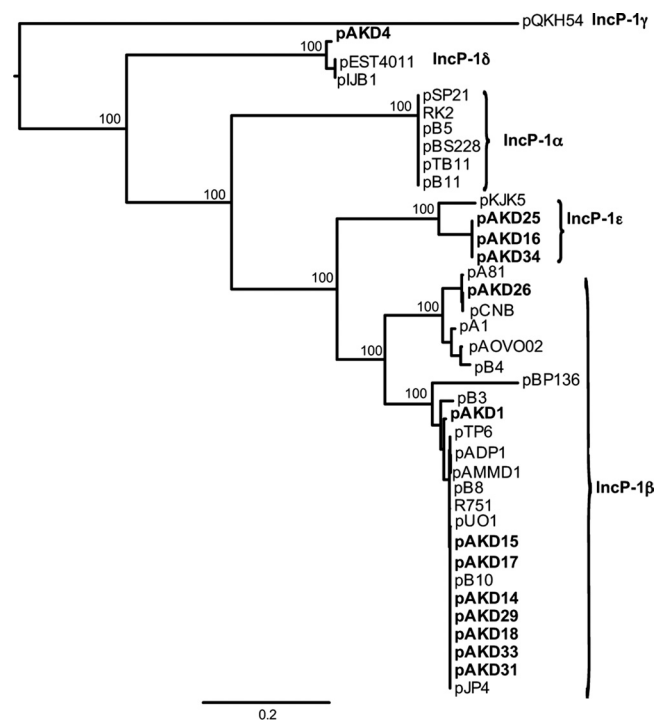


FIG. 1. Phylogenetic tree showing the relationship of the 12 pAKD plasmids described in this study and pAKD4 (27) (bold) with other completely sequenced IncP-1 plasmids. A maximum likelihood tree was inferred from nucleotide sequences of the relaxase gene, *traI*. Bootstrap values of the deep branches are shown to the left of each node. The scale bar represents the probability of nucleotide substitutions per site. Accession numbers for the plasmids used to construct the phylogenetic tree are given in Materials and Methods.



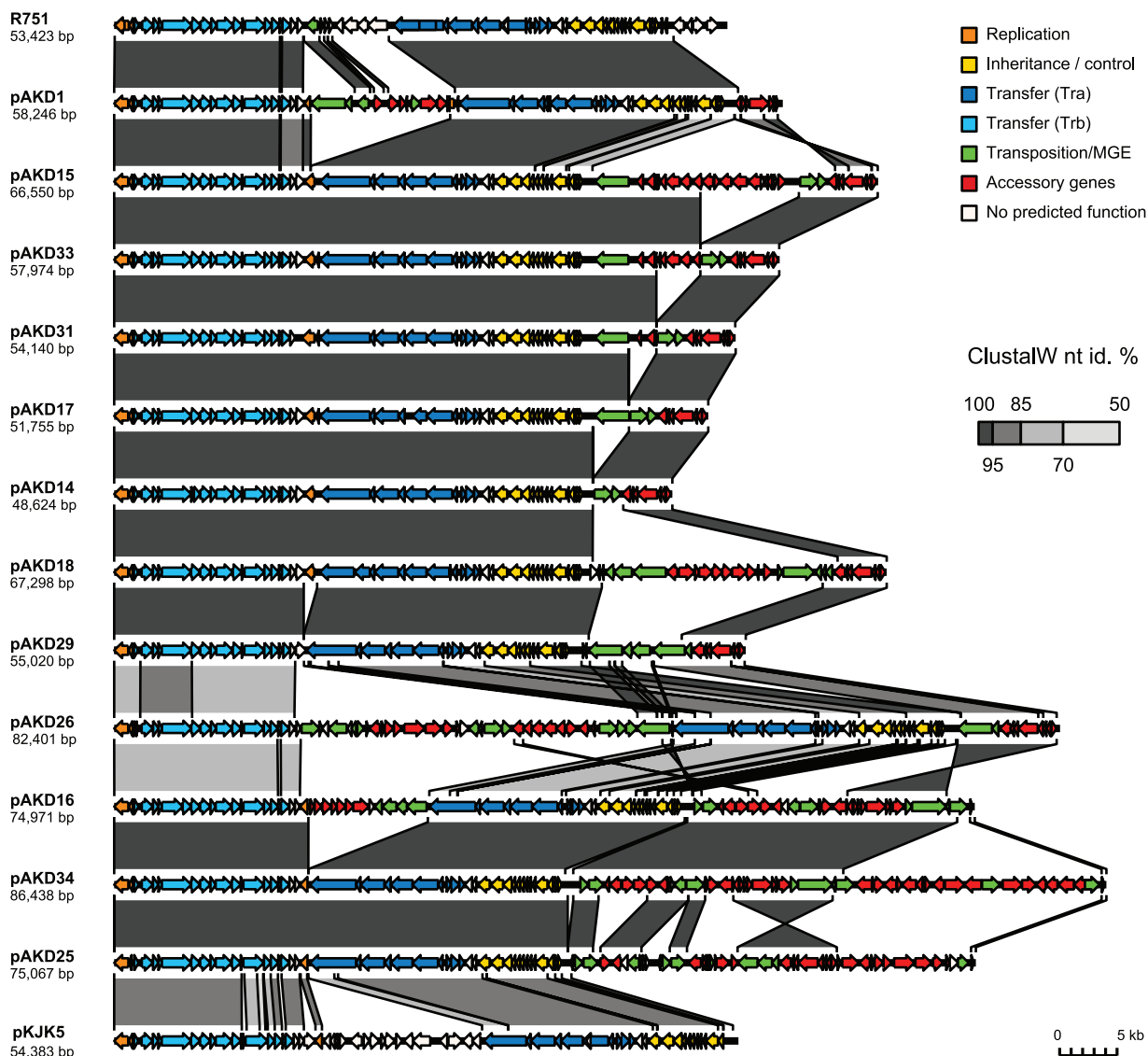


FIG. 2. Alignment of 12 pAKD plasmids showing their evolutionary relationship to each other and to the well-known IncP-1 plasmids R751 and pKJK5. Coding regions are shown as colored arrows; putative functions are indicated by the color key (top right). The degree of similarity between plasmids (percent nucleotide identity of alignments performed using ClustalW) is indicated by grayscale-shaded regions; the darker the shading between two segments, the higher their similarity, as shown in the heat key (middle right).

IncP-1 $\epsilon$  group. This subgroup is so far represented by only a single, completely sequenced, published plasmid, prototype pKJK5, and a partially sequenced catabolic plasmid, pEMT3 (2, 12, 37). The addition of these three new IncP-1 $\epsilon$  plasmid genome sequences increases the diversity of IncP-1 $\epsilon$  plasmids, since their backbone genes are considerably divergent in sequence from those of pKJK5 (Fig. 1 and 2).

Of the three IncP-1 $\epsilon$  plasmids, pAKD16 and pAKD34 are 100% identical in the entire backbone region except for their origins of replication (*oriV*). The *oriV* of pAKD34 appeared to have undergone a tandem duplication of 480 bp, resulting in 20 iterons instead of the 10 found on pAKD16. Since iterons are known to negatively regulate plasmid copy number (5) and have been directly implicated in IncP-1 copy number control (35), we tested whether pAKD34 had a lower copy number

than that of pAKD16 in *E. coli*. As a proxy for plasmid copy number, we determined the ratio of plasmid-carried *trbB* to chromosomal *atpB* in exponential phase by qPCR. However, these ratios were not significantly different ( $0.39 \pm 0.04$  for pAKD34 and  $0.38 \pm 0.06$  for pAKD16). The low values for both plasmids could be in part due to the presence of multiple chromosome replication forks in exponential phase, since the *atpB* gene is close to *oriC*. The findings suggest that the extra iterons of pAKD34 did not detectably affect its copy number.

**Deletions in accessory regions of five IncP-1 $\beta$  plasmids.** We found deletion variants when comparing the accessory regions between *oriV* and *trfA* of five IncP-1 $\beta$  plasmids, pAKD14, pAKD15, pAKD17, pAKD31, and pAKD33, which were isolated from the same soil and have identical backbones. As shown in Fig. 3, they all share the same

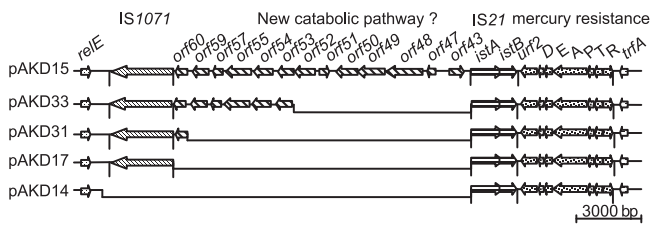


FIG. 3. Alignment of accessory regions of five pAKD plasmids indicating deletions of various sizes relative to pAKD15. Arrows represent ORFs showing direction of transcription. The plasmids shown here have 100% identity along their backbone sequences but have deletions in their accessory regions, shown as black lines. All deletions are flanked by IS21 at the 3' end, while sequences at the 5' end are variable. The mercury resistance genes are carried on a Tn501-like transposon that is not intact. Also shown here is the putative novel catabolic pathway of pAKD15 (Table 3). Intergenic regions are not drawn to scale.

IS21-like element with intact ends and a remnant of a Tn501-like mercury resistance transposon in this accessory region, yet they differ in the number of genes present upstream of these mobile genetic elements. Plasmid pAKD15 has the longest region, composed of an IS1071-like element and a cluster of genes forming a putative novel catabolic pathway, while the other four plasmids seem to be deletion derivatives of pAKD15. While the sequence flanking the deletion at one end is always conserved and corresponds to the right inverted repeat (IRR) of the IS21-like element, the other end is variable between plasmids (Fig. 3). We therefore hypothesize that the plasmids were generated from pAKD15 or a similar ancestral plasmid by DNA rearrangements due to intramolecular transposition of IS21. Since we consider these five plasmids to be variants of each other, we will from here on refer to the most complete plasmid, i.e., pAKD15, as the representative of this group.

**Deletions in the accessory region of IncP-1ε plasmids.** Similarly to the deletion variants found among the IncP-1β pAKD plasmids, we found a deletion variant among the IncP-1ε plasmids. The closely related plasmids pAKD16 and pAKD34 were isolated from the same soil (Ås) but using different procedures (triparental and biparental mating, respectively [Table 2]). The main differences between the plasmids are the following: (i) pAKD16 is missing nearly 20 kb of accessory genes present on pAKD34 between *oriV* and *trfA* and (ii) pAKD16 has a 10-kb transposon between the *tra* and *trb* regions that is not present on pAKD34. First, the loss of the 20-kb fragment may have occurred through recombination between two direct repeat sequences of *tnpA*<sub>IS<sub>PPs1</sub></sub> (*orf57* and *orf77* of pAKD34). It is more likely that the difference is a deletion in pAKD16 rather than an insertion in pAKD34 because the deleted region is part of a dichloropropionic acid degradation pathway. *De novo* addition of pathway genes is less likely than partial loss of an existing pathway. Second, the 10-kb transposon between *tra* and *trb* of pAKD16 is identical to Tn6048 in the genome of *Cupriavidus metallidurans* CH34. We postulate that, like plasmid pMOL98 (40), pAKD16 also captured Tn6048 from the recipient used in the triparental mating, which was strain AE815, a derivative of CH34. Thus, although exogenous plasmid isolation is an invaluable tool to obtain plasmids

from uncultured bacteria, the potential of gene exchange between plasmid and recipient genome should be considered.

**Mercury resistance transposons of the pAKD plasmids.** Mercury resistance genes were found on all 12 pAKD plasmids, as expected since all plasmids are known to confer mercuric chloride resistance (Table 2) (8, 9). The plasmids differ, however, in the transposons that carry the mercury resistance genes and in the location of these within the plasmid backbones. First, a Tn501 family transposon is located between *oriV* and *trfA* of the eight IncP-1β plasmids closely related to prototype R751 (pAKD1, pAKD15 and its four derivatives, pAKD18, and pAKD29); on pAKD1, however, the transposon may have inserted during an independent transposition event because of its reverse orientation and high nucleotide sequence divergence in the *mer* genes (7%). Second, another distinctly different Tn501 family transposon was found between *tra* and *trb* of the divergent IncP-1β plasmid pAKD26 and between *oriV* and *trfA* of the three IncP-1ε plasmids. The latter transposon differs from the former in the presence of an additional *mer* gene (*merC*) and high nucleotide sequence divergence (13%). These transposons were different from the Tn5053-like transposon found on the previously characterized IncP-1δ plasmid pAKD4 from the same plasmid collection (27). The different combinations of plasmid backbones and transposons clearly suggest various independent acquisition events.

**Hypothetical evolutionary pathway for the Tn501 group of IncP-1β plasmids.** The eight plasmids carrying the first type of Tn501-like transposon (all except pAKD1) are closely related to the four IncP-1β plasmids pB10, R906, R772, and pJP4 based not only on high nucleotide sequence identity (99 to 100%) in their backbone genes but also on the occurrence of a Tn501-like mercury transposon in exactly the same site (20, 25, 28). There are two possible hypotheses to explain this observation: (i) all plasmids share a recent common ancestor that already contained the Tn501-like transposon and, thus, that transposon inserted once into an ancestor of these plasmids, which subsequently gained different accessory genes, such as a Tn5393c transposon in pB10 or the *tfd* genes in pJP4, or (ii) the sequence between *oriV* and *trfA* in IncP-1β plasmids serves as a hot spot for the insertion of Tn501-like transposons, and this occurred multiple times in these seven plasmids. The high level of sequence identity found between the *merA* genes provides strong support for the first hypothesis. We therefore propose the following model for the evolution of some of these plasmids, as illustrated in Fig. 4. The ancestral IncP-1β plasmid with a Tn501-like insertion between *oriV* and *trfA* acquired an IS1071-related element in *tnpA*<sub>Tn501</sub>. This resulted in the formation of a pAKD29-like plasmid with 273 bp of the 3' end of *tnpA* removed from its 5' end. The pAKD29-like plasmid then underwent rearrangements to give rise to the following: (i) R906, through transposition of the streptomycin resistance carrying transposon Tn5393c into *tnpA*<sub>Tn501</sub>, at a site upstream of the previous insertion; (ii) pB10, formed from an R906-like plasmid by the acquisition of a tetracycline resistance transposon, Tn1721, which disrupted *tnpA*<sub>IS1071</sub>; (iii) pAKD29, formed from the pAKD29-like plasmid described above, through the acquisition of an IS21 family element within its IS1071-related element; and (iv) pAKD18, formed from the

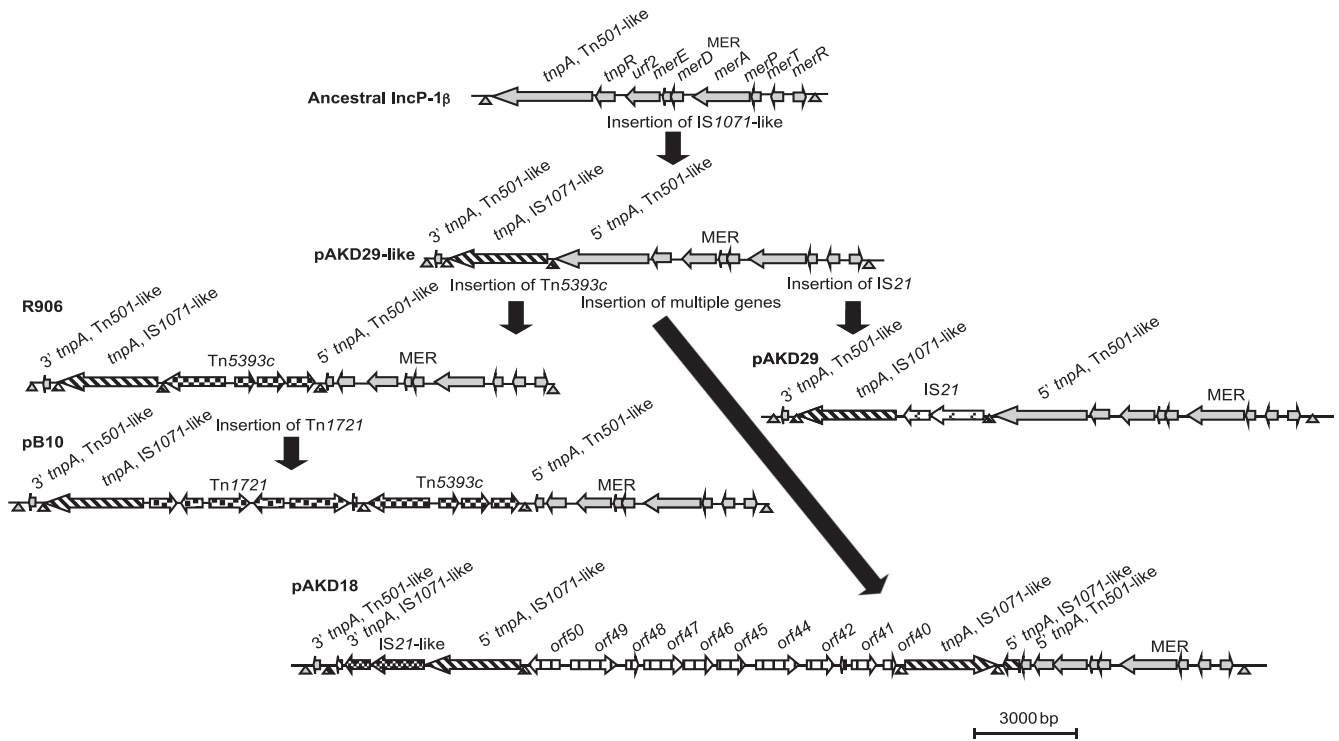


FIG. 4. Hypothetical evolutionary pathway for the Tn501 group of IncP-1β plasmids.

pAKD29-like plasmid by the insertion of multiple genes, *orf50* to *orf40*. Plasmid R772 was probably not formed from the pAKD29-like ancestral plasmid, since the 3' end of *tnpA*<sub>Tn501</sub> is much longer (638 bp) and does not have an *IS1071*-like element near it. Formation of R772 by the insertion and subsequent disruption of the Tn501-like element by multiple Tn21-like elements has been recently hypothesized (20). Thus, although R772 is very similar to R906, pB10, and pJP4, it may have been formed through a different pathway. Because plasmids pAKD15 and pJP4 are missing *tnpAR* genes of Tn501, depriving us of crucial information regarding the context of insertion, it is difficult to hypothesize their evolutionary pathway. In any case, such high backbone conservation among these plasmids is surprising, considering that the plasmids were isolated from different parts of the world over a period of 30 years. This could be explained by a very recent (but at least 30-year-old) worldwide spread of these plasmids, with insufficient time for multiple mutations to accumulate, or strong purifying selection acting on the entire plasmid backbone, preventing sequence divergence.

**Catabolic pathways encoded on four plasmids.** Four of the 12 pAKD plasmids contain multiple catabolic genes, even though none was isolated from soil based on the ability to confer degradation of an organic compound. In line with several previously published IncP-1 plasmids (26, 38), at least three plasmids encode gene products known to be involved in the degradation of chlorinated aromatic compounds: (i) 2,4-dichlorophenoxyacetic acid (2,4-D) degradation (*tfd* genes on pAKD25 and pAKD26), (ii) chlorocatechol degradation (*mocp* genes on pAKD26), (iii) dichlorophenoxypropionic acid (dichloroprop) degradation (*sdpA* and *rdpA* genes on pAKD34),

and (iv) a possible novel pathway of unknown function on pAKD15.

The two plasmids that carry 2,4-D degradation (*tfd*) genes, pAKD25 and pAKD26, were isolated from the same agricultural soil and yet belong to different IncP-1 subgroups, IncP-1ε and IncP-1β, respectively (Fig. 1). They carry these genes at different sites: between *oriV* and *trfA* on pAKD25 and between *trb* and *tra* on pAKD26. The identical gene organization and sequence identity of the *tfd* genes suggest that the two gene clusters are closely related to each other and to those found on the IncP-1δ plasmids pEST4011 (41) (Fig. 5) and pIJB1 (21)

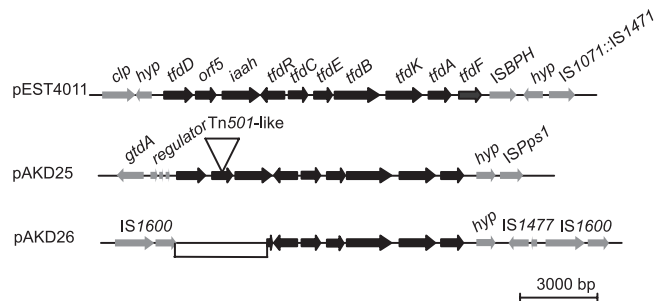


FIG. 5. Comparison of *tfd* gene regions from pAKD25 and pAKD26 with pEST4011. The black arrows show *tfd* genes, while the gray arrows show flanking sequences. The *tfd* gene order is conserved among all three plasmids, but pEST4011 has the most complete *tfd* gene cluster, while both pAKD25 and pAKD26 have been interrupted and are missing *tfdF*. *clp*, chloride channel protein; *hyp*, hypothetical protein; *gtdA*, gentisate 1,2-dioxygenase; *iaah*, indole acetamide hydrolase. Intergenic regions are not drawn to scale.

TABLE 3. Amino acid similarities to proteins in GenBank and domains in Interpro for ORFs of the putative pAKD15 catabolic pathway<sup>a</sup>

ORF no.	% amino acid similarity	Best hit, accession no.	Domain(s)	PFAM/Interpro no.
Orf60	86	Arsenical resistance protein, YP_002943872	NADPH-dependent FMN reductase, arsenate resistance	PF03358, IPR014063
Orf59	40	Protein of unknown function DUF81, YP_002947552	Protein of unknown function DUF81	PF01925
Orf57	31	Methanesulfonate monooxygenase, hydroxylase beta subunit, YP_001020117	Aromatic-ring-hydroxylating dioxygenase beta subunit	PF00866
Orf55	39	Putative dioxygenase subunit, YP_004417630	Aromatic-ring-hydroxylating dioxygenase alpha subunit, aromatic-ring-hydroxylating dioxygenase 2Fe-2S-binding site, Rieske iron-sulfur domain	IPR001663, IPR015881, IPR017941
Orf54	48	Periplasmic isophthalate binding receptor, BAH70270	<i>Bordetella</i> uptake gene	IPR0005064/PF03401
Orf53	43	Transcriptional regulator, IclR family, YP_003607133	Transcriptional regulator IclR N-terminal, winged HTH transcription repressor DNA-binding, transcriptional regulator IclR C terminal	PF09339/IPR005471, IPR011991, IPR014757
Orf52	68	Phthalate 4,5-dioxygenase, YP_002947550	NAD-binding domain, FAD-binding domain, 2Fe-2S cluster	PF00111
Orf51	35	Transcriptional regulator, MarR family protein, YP_002298705	Transcriptional regulator HTH MarR type, winged HTH transcription repressor DNA binding	IPR000835, IPR011991
Orf50	45	Extracytoplasmic solute receptor, YP_725900	<i>Bordetella</i> uptake gene	PF03401/IPR005064
Orf49	45	Rieske [2Fe-2S] domain protein, ZP_03542810	Rieske [2Fe-2S] iron-sulfur domain	PF00355/IPR017941
Orf48	44	FAD-dependent oxidoreductase, YP_973874	NAD(P)-binding domain, FAD/NAD(P)-binding domain	SSF51735, SSF51905
Orf47	34	ABC transporter, ZP_07673750	NMT1/THI5-like	PF09084
Orf43	39	GntR family transcriptional regulator, YP_675818	Transcriptional regulator HTH GntR, GntR C terminal, winged HTH transcription repressor DNA binding	IPR000524, IPR011711, IPR011991

<sup>a</sup> Abbreviations: FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; HTH, helix-turn-helix.

and less similar to the *tfdI* and *tfdII* clusters of the prototype 2,4-D catabolic plasmid pJP4. Both plasmids appear to lack *tfdF*, which encodes chloromaleylacetate reductase, the last plasmid-encoded step in the 2,4-D pathway. Moreover, the *tfd* clusters on both plasmids are disrupted by different insertions. On pAKD25, the insertion of a Tn501-like mercury transposon disrupted the open reading frame (ORF) between *tfd* and *iaah*. On pAKD26, an IS1600 element transposed into the *tfdR* end of the cluster, deleting a segment spanning *tfdD*, an uncharacterized ORF, and part of *iaah*. TfdD has been shown to be essential for the conversion of 2,4-dichloro-*cis,cis*-muconate to 2-chlorodiene lactone in the pJP4-encoded pathway. A simple growth assay indicated that plasmids pAKD25 and pAKD26 did not allow *C. pinatubonensis* JMP228n to utilize 2,4-D as sole carbon source when inoculated in liquid MMO supplemented with 2,4-D. No increase in turbidity was observed after 6 days, but both strains grew well in the same medium supplemented with acetate. In contrast, JMP228n(pEMT1::Km), previously shown to degrade 2,4-D (37), grew well with either acetate or 2,4-D. This lack of functionality of the *tfd* operons is likely due to the absence of *tfdF* from both plasmids and *tfdD* on pAKD26.

The IncP-1 $\beta$  plasmid pAKD26 has a second chlorocatechol degradation cluster which shows 99% nucleotide sequence identity to a corresponding region of *Bordetella petrii* strain DSM 12804 (AM902716) and plasmid pA81 (15). On pA81, this cluster is called *mocp* and is involved in chlorocatechol degradation. Based on the very high sequence, we also designated the pAKD26 genes *mocp*. The two chlorocatechol degradation clusters on pAKD26 (*tfd* and *mocp*) have no sequence similarity to each other, suggesting that they have different

evolutionary origins and were not generated by intramolecular duplication. Apparently, MocpB and MocpD were unable to substitute for the functionally equivalent proteins TfdD and TfdF, respectively.

The accessory region of pAKD34 between *oriV* and *trfA* contains genes that may be involved in degradation of the common herbicide dichlorprop, based on comparison with plasmid pMC1 from *Delftia acidovorans* (24). This region of pAKD34 is approximately 45 kb in size, of which a 20-kb segment shows 99% identity in nucleotide sequence to the corresponding sequence of pMC1. It encodes two dioxygenases, a transporter, a decarboxylase, a bacterioferritin, and a regulator. Just like on pMC1, the two dioxygenase-encoding genes, *rdpA* and *sdpA*, are also separated by a Tn501-like mercury resistance transposon that inserted into the *selA* gene downstream of *sdpA* on MC1, suggesting that the 20-kb fragments on the two plasmids share a recent common ancestor. A preliminary degradation assay showed no change in the concentration of dichlorprop or cell density in cultures of JMP228n(pAKD34) for at least 6 days, suggesting that the pathway is not functional in this host and needs further investigation.

The 24,640-bp-long accessory region of pAKD15 showed intermittent similarity when queried against nucleotide databases at NCBI, suggesting that this collection of genes had not been sequenced before. Additionally, preliminary characterization by similarity searches against the Interpro database showed that this region might encode a catabolic pathway. Components of this novel catabolic pathway are summarized in Table 3 (17). An identical pathway was also detected on another IncP-1 $\beta$  plasmid, pKSP18, isolated from the rhizosphere



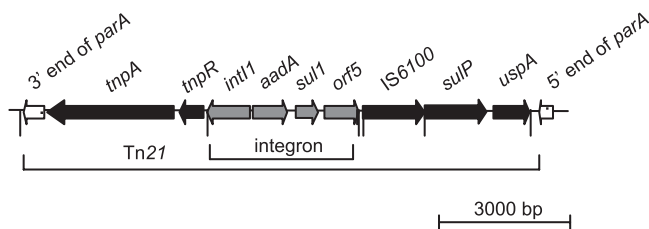


FIG. 6. Antibiotic resistance-bearing Tn21-like transposon of pAKD1. This 11,920-bp transposon disrupted the resolvase (*parA*), resulting in separation of the 3' and 5' ends. It consists of transposition genes *tnpA* and *tnpR*, a class 1 integron, IS6100, a sulfate transporter (*sulP*), and a universal stress protein (*uspA*). The integron has an integrase (*int1*), an aminoglycoside adenylyltransferase gene (*aadA*) encoding resistance to streptomycin and spectinomycin, a sulfonamide resistance gene (*sul1*), and a putative acetyltransferase (*orf5*).

of sugar beets in Wales, United Kingdom (K. Smalla, H. Heuer, and E. M. Top, unpublished data). The presence of these two plasmids isolated from different countries suggests that this gene cluster encodes a catabolic pathway and does not just represent a random collection of genes. Further studies should enable us to determine what catabolic compounds are degraded by this pathway.

Multiple catabolic pathways were found on the pAKD plasmids. Since each of the plasmids was isolated from agricultural soils treated with herbicides, these compounds may have selected for the catabolic pathways. Based on the limited information about the soil treatment histories (K. Drønen, personal communication), a link between the chemicals used and the catabolic pathways found on the plasmids is difficult to establish and therefore speculative. For example, plasmids pAKD25 and pAKD26 with the *tfI* genes were both isolated from soil Garpestad, a potato field likely treated with herbicides like Sencor (metribuzin, a triazinone) and Roundup (glyphosate), but also 2,4-D and related compounds such as 2-methyl-4-chlorophenoxyacetic acid (MCPA) have been widely applied in the past. Plasmids pAKD34, containing the dichlorprop degradation genes, and pAKD15, with the novel degradation pathway, were isolated from soil Ås, which was used to grow various crops, including cereals, and was likely treated with herbicides such as Roundup, Ramrod (propachlor or 2-chloro-*N*-isopropylacetanilide), and Banvel (also named dicamba or 3,6-dichloro-2-methoxybenzoic acid). These chlorinated aromatic compounds or their degradation by-products may well have been substrates for the putative catabolic pathways encoded on these plasmids.

**Plasmid pAKD1 encoding antibiotic resistance.** The only plasmid that encoded antibiotic resistance in addition to mercury resistance is the IncP-1 $\beta$  plasmid pAKD1. It confers resistance to 25 to 100  $\mu$ g/ml of spectinomycin, 25 to 50  $\mu$ g/ml of streptomycin, and up to 200  $\mu$ g/ml of the sulfonamide sulfathiazole to *E. coli* CV601. As shown in Fig. 6, this plasmid carries a multidrug resistance Tn21-like transposon with a class 1 integron. Thus, even though pAKD1 was isolated from an agricultural soil based on its ability to confer resistance to HgCl<sub>2</sub> only, it encodes several antibiotic resistance determinants, suggesting that mercury treatment might have coselected for the multidrug resistance genes. This would be in line with previous observations that pollu-

tion with heavy metals can cause a rise in the abundance of drug resistance genes (3).

In addition to our finding of new divergent IncP-1 $\epsilon$  plasmids, a recent study defined a sixth subgroup ( $\zeta$ ) represented by plasmids from a marine biofilm (18), and plasmids from *Neisseria gonorrhoeae* were also suggested to belong to a new subgroup (19). Thus, our and other studies are showing that the IncP-1 group of promiscuous plasmids is very diverse in both backbone sequence and backbone and accessory gene content. Further work in this field is needed to understand the extent and implications of the diversity and evolutionary history of the IncP-1 and other BHR plasmid groups.

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