Published in final edited form as:

Plasmid. 2010 March ; 63(2): 98. doi:10.1016/j.plasmid.2009.11.005.

Comparative genomics of pAKD4, the prototype IncP-1 δ plasmid with a complete backbone

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Abstract

Plasmids of the incompatibility group IncP-1 are important agents of horizontal gene transfer and contribute to the spread of antibiotic resistance and xenobiotic degradation within bacterial communities. Even though some prototype plasmids have been studied in much detail, the diversity of this plasmid group was still greatly underestimated until recently, as only two of the five currently known divergent sub-groups had been described. To further improve our insight into the diversity and evolutionary history of this family of broad-host-range plasmids, we compared the complete nucleotide sequence of a new IncP-1 δ plasmid pAKD4 to the genomes of other IncP-1 plasmids. Plasmid pAKD4 was previously isolated by exogenous plasmid isolation from an agricultural soil in Norway. Its 56,803 bp nucleotide sequence shows high similarity in gene sequence and gene order to both plasmids pEST4011 and pIJB1, the only other IncP-1 δ plasmids sequenced so far. While all three plasmids have a typical IncP-1 backbone comprising replication, transfer and stable inheritance/control genes, the low sequence similarity in some regions and presence/absence of some backbone genes compared to other IncP-1 plasmids cluster them in a divergent sub-group. Therefore this study validates the presence of a real IncP-1 δ clade with multiple plasmids. Moreover, since both pEST4011 and pIJB1 are missing a portion of their transfer genes, pAKD4 represents the first completely sequenced self-transferable plasmid with a complete IncP-1 δ backbone. We therefore propose it to be the prototype IncP-1 δ plasmid.

Keywords

Plasmid; horizontal gene transfer; broad host range; transposon; IncP-1 plasmid; mercury resistance

1. Introduction

Horizontal gene transfer plays a critical role in bacterial evolution and adaptation [1], and plasmid-mediated conjugation is thought to be one of the most common mechanisms of gene transfer between distantly related bacteria [2,3]. Of particular interest in this respect are broad-

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host-range (BHR) plasmids, which can transfer to and replicate in a wide range of bacterial hosts. They typically belong to incompatibility groups IncP, IncN, IncW and IncQ [4,5], and the recently defined PromA group [6].

The IncP group is considered to contain the most promiscuous plasmids, able to transfer to and replicate within different sub-groups of the Proteobacteria (α -, β -, γ -). From here on we will refer to them as IncP-1 plasmids, based on the *Pseudomonas* classification system. In addition to their wide replication range they can even mobilize IncQ plasmids and so-called 'shuttle vectors' to Gram-positive bacteria [7], cyanobacteria [8], or even Eukaryotes such as yeast [9]. Like all plasmids, those belonging to this group have two distinct regions [10]; the "backbone" region, which encodes functions involved in replication, transfer, and maintenance and control of the plasmid, and the "accessory" region consisting of genes that confer specific beneficial traits to the bacterial host such as antibiotic or heavy metal resistance, or the ability to metabolize xenobiotic compounds. Accessory genes are almost always associated with transposable elements and found in one or two sites i.e., between *oriV* and *trfA* and/or between the two transfer operons *tra* and *trb*. Plasmids with almost identical backbones can have entirely different accessory genes [11].

During the last five years, the IncP-1 plasmid group has been classified into five major phylogenetic sub-groups (IncP-1 α -, β -, γ -, ϵ -), based on phylogenetic analysis of the amino acid sequences of the replication protein TrfA, and a few other essential backbone proteins (12, 13, 14, 15, 16). While most of the backbone genes are shared among the different sub-groups, there are differences in gene composition and sequences that allow them to be distinguished (H. Suzuki et al., unpublished). Thus, while the architecture of IncP-1 plasmid genomes is conserved, there is significant divergence in many of the backbone genes and substantial variation in the accessory elements.

Despite the assumption that IncP-1 plasmids are crucial to the adaptive evolution of bacteria, their extant diversity is probably still underestimated. For example, for each of the γ -, δ -, and μ - sub-groups, only one plasmid has been completely sequenced and described so far [12,13, 16], although it was recently suggested that representatives of all five sub-groups were present in a single wastewater sample [17]. Moreover, little is known about the diversity of the mobile genetic elements they carry and the functions they encode [17,18]. Since some accessory genes on these plasmids have no homologues and cannot be assigned a function, it is not known what phenotypes other than the well-known resistance and degradation functions these plasmids may encode.

The goal of our study was to further describe the diversity and evolutionary history of IncP-1 plasmids by comparing the genome sequence of the IncP-1 δ plasmid pAKD4 to those of its closest relatives. Plasmid pAKD4 was isolated from Norwegian soil by Drønen et al [19] using a biparental exogenous isolation method, also referred to as plasmid capture, with selection for resistance to mercury chloride. The authors showed it to be a BHR mercury resistance (Hg^R) plasmid by its ability to conjugatively transfer to strains of the γ - and β -Proteobacteria when selection for Hg^R was imposed [19]. Based on the lack of hybridization with probes used in replicon typing for the known incompatibility groups [20], the plasmid was thought to belong to a new BHR group. However, its complete sequence obtained in this study revealed that its backbone is very similar to plasmid pEST4011 from Estonia, the first and only IncP-1 δ plasmid described so far [16]. Here we show that this IncP-1 δ clade now includes two other plasmids besides pEST4011: pAKD4 from Norway, and pIJB1, isolated in Scotland [21,22]. The latter had been reported earlier as an IncP-1 β plasmid, but its complete sequence (available in GenBank since April 2005, accession no. DQ065837) has now made it possible to infer its evolutionary history. We conclude that pAKD4 and pIJB1 are new members of the IncP-1 δ sub-group. Since pAKD4 is the only plasmid of the three with a complete set of typical

IncP-1 δ - like transfer genes and is self-transferable, we propose it to be the prototype plasmid for the IncP-1 δ subgroup.

2. Materials and Methods

2.1. Host range studies

Plasmid pAKD4 was isolated from an agricultural soil in Norway as part of a set of plasmids that were captured by biparental and triparental matings [19]. K. Drønen kindly provided this plasmid to us in *Pseudomonas putida* UWC1. The host range of plasmid pAKD4 was tested to confirm previous results [19]. The donor strain, *P. putida* UWC1 carrying plasmid pAKD4 was grown in Luria-Bertani broth (LB) medium, supplemented with 10 $\mu\text{g ml}^{-1}$ mercuric chloride (HgCl_2). Recipients used were as follows: *P. putida* KT2442::gfp, *Escherichia coli* CV601::gfp, *Cupriavidus necator* JMP228::gfp, and *Agrobacterium tumefaciens* UBAPF2::gfp [23]. Since all recipients are resistant to kanamycin (Km), they were grown in LB supplemented with 50 $\mu\text{g ml}^{-1}$ Km. The following mating protocol was used: One ml of overnight grown donor and recipient cultures were centrifuged and the cell pellets were re-suspended in 100 μl of LB broth. 50 μl of donor culture was mixed with 50 μl of recipient culture and plated on LB agar (LBA). After 18 hours of incubation at 30°C, the mating mixture was re-suspended in saline; diluted up to 10^{-5} and 100 μl were plated onto selective media to distinguish transconjugants (LBA with HgCl_2 and Km), recipients (LBA with Km) and donors (LBA with HgCl_2). Donors and recipients were also separately incubated on LBA and subsequently plated on selective media (LBA with HgCl_2 and Km or Gm) as negative controls. The presence of pAKD4 in transconjugants was confirmed after plasmid extraction from randomly chosen colonies using the Plasmid Mini kit (QIAGEN, Valencia, CA) and gel electrophoresis [24].

2.2. Plasmid DNA isolation

Plasmid DNA to be used for sequence determination was isolated from UWC1 (pAKD4) using the Plasmid Midi kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions for low-copy-number plasmids. Subsequent DNA preparations for additional analyses were obtained using the alkaline lysis method [24]. Standard methods were used for restriction analysis and gel electrophoresis [24].

2.3. Sequencing and Annotation

The DNA sequence of plasmid pAKD4 was determined by shotgun sequencing at the DOE Joint Genome Institute (Walnut Creek, CA). Briefly, after construction of ~3kb clone libraries, Sanger technology was used to determine the DNA sequences of 384 clones in both directions. These sequences were then assembled using PGA [25], and gaps were closed by primer walking. Automatic annotation was carried out by the JCVI (J. Craig Venter Institute) Annotation Service (<http://www.jcvi.org/cms/research/projects/annotation-service/overview/>) followed by manual annotation by the authors. The complete sequence of pAKD4 has been deposited in the GenBank database under the accession number GQ983559.

2.4. Bioinformatics analyses and software

GenBank (<http://ncbi.nlm.nih.gov>) was searched for similar sequences using BLAST [26]. Nucleotide and amino acid sequences were aligned using ClustalW [27]. Blastp searches of all hypothetical proteins in pAKD4 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 figure of the plasmid map was generated using GENtle

(<http://gentle.magnusmanske.de/>) open software package. The alignment figure of pAKD4 with pEST4011 and pIJB1 was generated using Mauve [28], ClustalW and PlasmidG (Van der Auwera, unpublished). To infer the phylogenetic relationship of pAKD4 with other sequenced IncP-1 plasmids, amino acid sequences of 20 backbone proteins shared by 22 IncP-1 plasmids were individually aligned and then concatenated into a single alignment. A maximum likelihood phylogeny based on this concatenated alignment was inferred using RAxML [29]. The 20 proteins were: TrfA, TrbA, TrbB, TrbC, TrbD, TraE, TraF, TraG, TraI, TraJ, TraL, TraM, KfrC, KfrB, KfrA, KorB, IncC, KleE, KorC, and KlcA.

Accession numbers of the IncP-1 plasmids included in this study are as follows: pQKH54 (NC_008055), pEST4011 (NC_005793), pIJB1 (DQ065837), 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), and pB8 (NC_007502).

3. Results and Discussion

3.1. General genome features of plasmid pAKD4

To improve our insight into the evolutionary history of IncP-1 plasmids, the complete nucleotide sequence of the circular plasmid pAKD4 was analyzed. General sequence features of plasmid pAKD4 revealed the following: the genome is 56,803 bp long and its G+C content 62.7%, which is similar to other IncP-1 plasmids. Moreover the G+C content was rather uniformly distributed across the genome (Table 1). The genome has a total of 63 ORFs and a few intergenic regions (Fig. 1). Of the 63 ORFs, seven could not be assigned a function (*orf18*, *orf19*, *orf46*, *orf47*, *orf58*, *orf61*, *orf63*). Some of these unknown genes are located within the backbone region of pAKD4, while others are in the accessory region (Fig. 1, Table 1). The predicted protein products of only two of the seven ORFs have conserved domains (*orf18* and *orf63*). Orf18 has a thermonuclease domain and has similarity to the staphylococcal nuclease family. Blastp searches showed 62% similarity to a hypothetical protein from *Collimonas fungivorans*, plasmid pTER33 [YP_001672028] (Table 1) where the coding sequence has been described as *parB*, involved in plasmid partitioning. Orf63 has a dimeric alpha-beta barrel domain. Since domains with this structure are found in proteins from several different families, it is hard to predict the function of this protein given only this domain information. However, blastp search against the non-redundant protein database at NCBI showed that this protein is 100% identical to hypothetical protein Rmet_6340 from *Ralstonia metallidurans* CH34 [YP_582149], which has been described to have a chlorite dismutase domain. Clearly, further work is needed to determine the phenotype of these hypothetical proteins.

The pAKD4 genome showed highest similarity in the backbone region with plasmids pEST4011 [16] and pIJB1 [21,22] (Fig. 2). Plasmid pEST4011 had been previously reported to belong to a new IncP-1 sub-group, called IncP-1 δ [16], but was the only reported member thus far. Plasmid pIJB1 had been reported to be an IncP-1 β plasmid based on replicon typing [22]. Sequence comparison of the backbone genes of pAKD4 to those of pIJB1 and pEST4011 shows between 98 and 100% nucleotide sequence identity. We therefore conclude that plasmids pAKD4 and pIJB1 are also members of this divergent IncP-1 δ sub-group. This validates the existence of a real IncP-1 δ clade with multiple members. Detailed comparison of the three backbones is described below (3.2.).

As in other IncP-1 plasmids, accessory genes of pAKD4 were carried on transposons, which were inserted in two regions i.e., between *oriV* and the *trfA* gene and between the *tra* (conjugative transfer) and *trb* (mating-pair formation) operons (Fig. 1). Details of coding

sequences and their translated protein sequence identity to corresponding homologues in the two accessory gene regions are listed in Table 1 and discussed in section 3.3.

3.2. Comparative analysis of the backbone regions of pAKD4, pEST4011 and pIJB1

The backbone region of pAKD4, comprised of replication (*oriV*, *trfA*), transfer (*tra* and *trb*) and stable inheritance/control (*ctl*) modules, is 31 kb long. As shown in Fig. 2 and Table 1, the first 5 kb of pAKD4 containing the genes *trfA*, *trbA*, *trbB*, *trbC*, *trbD* and part of *trbE* are 99% identical to pEST4011 and pIJB1. The remaining *trb* genes of pAKD4 (*trbF*, *trbG*, *trbH*, *trbI*, *trbJ*, *trbK*, *trbL*, *trbM*, *trbN*, *trbO*, *trbP*), as well as the 3' terminal regions of *trbE* and *traC* bear much lower similarity to pIJB1 and are simply missing on pEST4011. In the latter plasmid, the genes were probably deleted, as previously suggested [16] (Fig. 2, Table 1). In pIJB1, similarity is disrupted due to a probable recombination event, which introduced the pJP4-like genes *trfA-trbP* downstream of *trbE* (Fig. 2). Indeed, this region of pIJB1 shares higher nucleotide identity (99%) with the distantly-related IncP-1 β plasmid pJP4 [30] than with its close relative pAKD4 (70% identity; Table 1). This recombination event probably took place in the 3' end of *trbE*, close to where pEST4011 underwent a deletion of many of its transfer genes. Thus, plasmid pIJB1 has two sets of *trfA-trbE* genes, one of which seems native to the IncP-1 δ plasmid backbone and another that was likely acquired by recombination (Fig. 2). Additionally, the set of genes acquired by pIJB1 also includes intact *trfA1* and *ssb* genes, which may have complemented the truncated versions found on IncP-1 δ plasmids (see 3.2.1 below). Thus, in spite of the fact that plasmid pIJB1 is a product of recombination between an IncP-1 δ and an IncP-1 β plasmid, we are designating it an IncP-1 δ plasmid since 82% of its backbone genes are IncP-1 δ specific.

The rest of the backbone of pAKD4 coding for transfer genes (*traD*, *traE*, *traF*, *traG*, *traH*, *traI*, *traJ*, *traK*, *traL* and *traM*) and inheritance/control (*ctl*) genes (*kfrC*, *kfrB*, *kfrA*, *korB*, *incC*, *korA*, *kleF*, *kleA*, *kleE*, *korC*, *kfcA*, *kfcB*) have between 98 and 100 percent nucleotide sequence identity to their homologues on pEST4011 and pIJB1 and much lower identity (70%) to other IncP-1 plasmids (Fig. 2, Table 1). Of interest in this context is the remarkable gene order conservation in the *ctl* region (more specifically *kfrA-kfcA*) between the IncP-1 δ plasmids and plasmids of a recently defined clade within the IncP-1 β sub-group, including pA81 (NC_006830), pCNB1 (NC_010935), pB4 (NC_003430) and the recently sequenced plasmid pWDL7::rfp (Król et al., unpublished data) (data not shown). This gene order is also conserved in plasmids pB10 (NC_004840) and pJP4 (NC_005912), which likely resulted from recombination in that region between a pB4-like plasmid and another IncP-1 β plasmid [11]. Further research is required to determine the importance of recombination in IncP-1 plasmid evolution. In conclusion, pAKD4 is the only plasmid known so far to contain a complete typical IncP-1 δ plasmid backbone and should therefore be used as the prototype of this IncP-1 sub-group.

3.2.1. The replication initiation protein TrfA—An interesting feature of the IncP-1 δ plasmids is the replication initiation gene *trfA*. In most IncP-1 plasmids known so far this gene has two in-frame translational start codons, and thus encodes a long TrfA1 and a shorter TrfA2 protein (also called TrfA-44 and TrfA-33 in IncP-1 α plasmid RK2) [31]. Interestingly, since all three IncP-1 δ plasmids are missing the 5' end of *trfA*, which encodes the start codon for and the N-terminal region (350 bp) of TrfA1, TrfA2 is the only replication initiation protein encoded by these plasmids. The 3'-end of the *ssb* gene, which is usually found upstream of *trfA* in most IncP-1 plasmids, is also missing in these plasmids. This gene codes for a single strand DNA-binding protein, which helps in DNA replication, recombination and repair. TrfA1 and Ssb proteins play a crucial role in plasmid replication in some bacterial hosts such as *P. aeruginosa* and may not be needed in some others such as *P. putida* and *E. coli* [32]. Therefore we postulate that IncP-1 δ plasmids co-evolved with hosts that do not require TrfA1 and Ssb

proteins and thereby contracted their host range. Further research is needed to test this hypothesis.

3.2.2. Detection of IncP-1 δ specific genes—Some of the unknown ORFs on pAKD4 were also found on pEST4011 and pIJB1. They include *orf58*, found upstream of *klcA*, and *orf46* and *orf47*, between *kfrA* and *kfrB* (Fig. 1 and Table 1). The location of the latter two genes in the *kfrABC* operon suggests a possible involvement in stable inheritance.

3.2.3. Other similarities and differences among the backbones of pAKD4, pEST4011 and pIJB1—High conservation of sequence was found among the three IncP-1 δ plasmids in the origins of replication (*oriV*) and transfer (*oriT*), and in the binding sites of regulatory proteins (KorA, KorB, KorC and TrbA). Minor differences among the sequences were noted primarily in the form of indels of 1, 3 and 6 bp in *kfrC*, *traK* and *traI* respectively, causing a frameshift in the first case, and amino acid deletions or insertions in the other cases over a small segment of the protein. The effect of these differences on the plasmid phenotypes is currently unknown.

3.2.4. Phylogenetic analysis of plasmid pAKD4—To confirm that pAKD4 is an IncP-1 δ plasmid, a maximum likelihood tree was inferred from concatenated sequences of 20 backbone proteins shared by all IncP-1 plasmids and pAKD4 (Fig. 3). Note that none of the *trbF-P* genes missing in pEST4011 were included in this list. Also, of the two sets of *trbABCD* genes on pIJB1, only the ones having 99% identity to pAKD4 and pEST4011 were used. This tree shows that the three IncP-1 δ plasmids share a common ancestor and form a monophyletic group.

3.3. Transposable elements in pAKD4

In spite of the high similarity in plasmid backbone gene content, sequence and synteny between plasmid pAKD4 and plasmids pEST4011 and pIJB1, their accessory genes and the transposons that carry them are very different. Plasmids pEST4011 and pIJB1 both encode 2,4-dichlorophenoxyacetic acid (2,4-D) on transposons and pIJB1 potentially also biphenyl degradation (Fig. 2). In contrast, plasmid pAKD4 does not encode 2,4-D or biphenyl degradation determinants. It is the smallest of the three IncP-1 δ plasmids and has relatively few accessory genes (Fig. 2). These accessory genes are described below.

3.3.1. A Tn5053-like transposon that encodes mercury resistance—A mercury resistance Tn5053-like transposon was found upstream of the *parA* locus, between the *tra* and *trb* regions. This location seems to be a ‘hot-spot’ for insertion of such transposons, as shown in previous studies [33,34] (Fig. 4a). The family of Tn5053-like transposons is bounded by 25 bp inverted repeats and has 4 transposition genes (*tniA*, *tniB*, *tniQ*, *tniR*) [35] along with mercury resistance genes: *merR*, *T*, *P*, *C*, *A*, *D*, *E*. These predicted gene products are listed in Table 1. This transposon has 92% identity to the Tn5053-like transposon Tn502 of plasmid pVS6 from *P. aeruginosa* [36]. The 5-bp direct repeats typically generated upon transposition of Tn5053-like elements could not be detected at the extremities of this transposon, suggesting that DNA rearrangements may have taken place since transposition. The presence of mercury resistance determinants on pAKD4 was expected since Drønen *et al.* [19] showed that pAKD4 confers mercury resistance in several bacterial hosts.

3.3.2. A Tn3-like transposon that encodes a putative post-segregational killing system—The transposon inserted between *oriV* and *trfA* is a class II (Tn3-like) transposon that carries typical transposase and resolvase genes (*tnpA*, *tnpR*), potential toxin-antitoxin genes (*orf61*, *relE/stbE*), and a gene with unknown function (*orf63*) (Fig. 4b). This 5,461 bp transposon is 99% identical to its homolog of the same size on chromosome 1 of *R. picketti*

12J (GenBank accession no. NC_010682) and is 99% identical to only the first 4,497 nucleotides of the 5,836 bp Tn5501-related transposon on plasmid pB8, which includes *tnpA*, *tnpR*, *orf61* and *relE/stbE* [37]. Thus, while this entire transposon is completely conserved in *R. pickettii* 12J it has homology to only a part of the transposon of pB8. The latter transposon includes the *tnpA* and *tnpR* genes along with a putative addiction module toxin gene (*relE/stbE*) and two conserved hypothetical proteins. One of these was hypothesized to form a toxin-antitoxin pair with *relE/stbE*, thus representing a possible post-segregational-killing system on this plasmid. Based on sequence similarity, *orf61* and *relE/stbE* on pAKD4 may thus also form a toxin-antitoxin pair, but its function needs to be confirmed. The remaining ORF on the pAKD4 transposon, *orf63*, is of unknown function and identical to the corresponding ORF on chromosome 1 of *R. pickettii* 12J. The transposon is bordered by 38 bp-inverted repeats typical of Tn3-like transposons. A 5 bp target site (TCATC) duplication created due to the insertion of the transposon is still intact, suggesting that the insertion occurred recently.

3.4. Evolutionary history of the IncP-1 δ plasmids

Now that we have compared the backbones and accessory elements of the three IncP-1 δ plasmids known so far, the putative evolutionary mechanisms that led to these three plasmids, and their effects on the plasmid phenotypes and ecology can be discussed. All three have been isolated from soils in Europe, and the only other indication of the presence of IncP-1 δ plasmids is also from Europe (Denmark) [17]. Plasmid pAKD4 was isolated by a plasmid capture method from agricultural soil in Norway, and its host in that soil is not known [19]. pEST4011 was found in a 2,4-D degrading strain, *Achromobacter denitrificans* EST4011, isolated from agricultural soil in Estonia [16], and pIJB1 was isolated from another 2,4-D degrading strain, *Burkholderia cepacia* 2a, from soil in Aberdeen, Scotland [21]. Future studies should determine the geographic distribution of these plasmids worldwide.

It is striking that both the deletion in pEST4011 and the recombination in pIJB1 occurred in the *trfA-trb* region, specifically in *trbE*. Short direct repeats (AGCGCCCTG) may have been responsible for these events, as they are found in *trbE* of all three IncP-1 δ plasmids - flanking the deleted and inserted regions of pEST4011 and pIJB1 respectively, as well as in *traC* of plasmid pAKD4. Recombination between short direct repeats followed by deletion of the sequence between them is a common mechanism of gene loss [38]. While the deletion in pEST4011 made it non-self-transferable, it is not clear if the transferability and host range of pIJB1 was affected by the putative recombination with a pJP4-like plasmid (see Fig. 2 and section 3.2). This plasmid has been shown to transfer its 2,4-D degradation and mercury resistance phenotypes, but to our knowledge the plasmid's host range has not been examined (I.J. Bruce, personal communication). Whether or not *trbE* is a 'hot-spot' for recombination in these plasmids will become clear once more IncP-1 δ plasmid genome sequences are available.

Despite DNA rearrangements, the shared backbone genes of the three IncP-1 δ plasmids are very conserved in sequence (98-100%). Sequence comparisons show higher similarity between pIJB1 and pEST4011 than between either of these two plasmids and pAKD4, suggesting that pIJB1 and pEST4011 share a more recent common ancestor. Evolution of this IncP-1 δ sub-group might thus have taken place as follows. An ancestral IncP-1 δ plasmid gave rise to the common ancestor of pEST4011 and pIJB1, and to pAKD4. Transposons carrying *tfd* genes may have been acquired by pEST4011 and pIJB1 independently, since they are in reverse order in the two plasmids. Plasmid pAKD4 likely evolved independently by acquiring a mercury resistance bearing Tn5053-like transposon and a toxin-antitoxin system bearing Tn3-like transposon.

In the course of their evolutionary history, the IncP-1 δ plasmids have gained and lost certain features that make this set of plasmids unique. One such feature is the absence of the N-terminal

region of the TrfA protein, which has been hypothesized to play a role in the evolution of host range in these IncP-1 δ plasmids (see also section 3.2.1). Other features include the absence of *upf30.5* and *upf31.5*, located downstream of *trbP* on most IncP-1 β and IncP-1 ϵ plasmids. Certain genes found in the *ctl* region of several other IncP-1 plasmids such as *kleB*, *kleC*, *kleD*, *kleG*, *klaA*, *klaB*, *klaC*, *kluA*, *kluB*, *korF*, *korG* were not found on pAKD4, pEST4011 and pIJB1. In contrast, *orf46*, *orf47*, and *orf58* seem to be specific to the IncP-1 δ sub-group, as they do not have homologues on other IncP-1 plasmids (see also 3.2.2.). Thus a survey of the IncP-1 δ plasmids reveals several ORFs that are conspicuously absent and a few that are specific to this group. Future work will have to determine the impact of the presence and absence of these genes in the *ctl* region on stable plasmid inheritance and possibly other plasmid features.

3.5. Plasmid host range

To confirm previous findings that plasmid pAKD4 can transfer and replicate in γ - and β -Proteobacteria, but not in α -Proteobacteria [19], the host range of pAKD4 was examined again. We showed transfer of pAKD4 from its laboratory host *P. putida* UWC1 (γ -Proteobacteria) to *P. putida* KT2442::*gfp* (γ -Proteobacteria), *E. coli* CV601::*gfp* (γ -Proteobacteria) and *C. necator* JMP228 (β -Proteobacteria), but transfer to *Agrobacterium tumefaciens* UBAPF2::*gfp* (α -Proteobacteria) could not be detected (data not shown). This is in agreement with previous results [19] and confirms that pAKD4 is a broad-host-range plasmid, albeit with a narrower host range than most other IncP-1 plasmids. Future rigorous host range studies will be needed to determine to what extent truncation of *ssb* and *trfA* in the IncP-1 δ plasmids, or other missing backbone genes, caused a contraction of their host range, and if natural recombination events like the one that likely resulted in plasmid pIJB1, can reverse that host range contraction.

3.6. Conclusions

Based on previous studies [16] and our work, it can be concluded that IncP-1 δ plasmids have diverged considerably from other IncP-1 plasmids and share some genomic features that might have an impact on the range of hosts in which they can stably persist. Future studies that characterize IncP-1 and other BHR plasmids will further increase our understanding of the diversity and evolutionary history of these important agents of horizontal gene transfer in prokaryotes. Together with comparative host range studies they will reveal evolutionary mechanisms that allow IncP-1 plasmids to modulate their host range.

Acknowledgments

We would like to thank Karine Drønen for providing us with plasmid pAKD4 and Kornelia Smalla for sending us the recipient strains used in host-range assays. We are also grateful to Brian Foster at the DOE Joint Genome Institute (JGI) for help with sequencing this plasmid, and to Geraldine Van der Auwera for drawing Figure 2. This work was funded by the National Science Foundation Grant EF-0627988 and by the National Institutes of Health NCRR grants P20RR16448 and P20RR016454.

Abbreviations

BHR	broad host range
CDS	coding sequence
MGE	mobile genetic elements

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Figure 1. Genetic map of pAKD4. Coding regions are illustrated by arrows showing the direction of transcription. The different functional modules of the plasmid are represented in different colors. In addition, the origins of vegetative replication (*oriV*) and transfer (*oriT*) are shown as black circles.

Figure 2.

Alignment of the IncP-1δ plasmids showing their evolutionary relationship. Coding regions are shown as colored arrows; putative functions are indicated by the color key below the alignment. The degree of similarity between plasmids (nucleotide identity percentage 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 (top right). The overlap of shaded regions resulting from the duplicated *trfA-trbE* segment on pIJB1 is shown with a transparency effect for optimal visibility of the different segments and their respective similarities. The figure shows that pAKD4 is the only IncP-1δ plasmid that has an intact backbone, since pEST4011 has a large deletion and pIJB1 has one set of pJP4-like IncP-1βs backbone genes. Regions surrounding these areas are very conserved among all three plasmids.

Figure 3.

Phylogenetic tree showing the relationship of pAKD4 with other IncP-1 plasmids. Maximum Likelihood tree was inferred from concatenated amino acid sequences of 20 backbone proteins shared by completely sequenced IncP-1 plasmids and pAKD4. The scale bar represents the probability of amino acid substitutions per site. References for the plasmids used to construct the phylogenetic tree are given in the Materials and Methods section.

Figure 4.

Accessory regions of pAKD4. a) Tn5053-like transposon carrying transposition genes (*tniA*, *B*, *Q*, *R*) and mercury resistance genes (*merR*, *T*, *P*, *C*, *A*, *D*, *E* and *urf2*), inserted between *tra* and *trb* b) Tn3-like transposon carrying putative toxin-antitoxin genes (*orf61* and *relE*) and an ORF of unknown function (*orf63*) inserted between *oriV* and *trfA*. The rectangles represent inverted repeats and the ovals represent direct repeats. The Tn3-like transposon has both inverted and direct repeats.

Table 1

Summary of all coding sequences of pAKD4 including start/stop positions, protein length, G+C%, gene product names, accession numbers of best blast hits and their corresponding percent identity.

CDS#	Gene name	Start	Stop	Size of protein (aa)	Strand	GC(%)	Product	Most closely related gene product and its Genbank accession number	% aa Identity
1	<i>trfA2</i>	1	870	290	-	60	replication initiation protein	TrfA [<i>Achromobacter denitrificans</i> , pEST4011] NP_990917	99
2	<i>trbA</i>	1191	1568	126	+	54	mating pair formation protein, transcriptional regulator	TrbA [<i>Achromobacter denitrificans</i> , pEST4011] NP_990918	98
3	<i>trbB</i>	1823	2785	321	+	60	mating pair formation, ATPase	TrbB [<i>Achromobacter denitrificans</i> , pEST4011] NP_990919	100
4	<i>trbC</i>	2797	3225	143	+	62	mating pair formation prepin	TrbC [<i>Achromobacter denitrificans</i> , pEST4011] NP_990920	100
5	<i>trbD</i>	3228	3539	104	+	58	mating pair formation protein	TrbD [<i>Achromobacter denitrificans</i> , pEST4011] NP_990921	100
6	<i>trbE</i>	3536	6073	846	+	60	mating pair formation NTPase	TrbE [Uncultured bacterium, pKJK5] YP_709146	87
7	<i>trbF</i>	6070	6846	259	+	63	mating pair formation protein	TrbF [Uncultured bacterium, pTB11] YP_112373	74
8	<i>trbG</i>	6859	7764	302	+	63	mating pair formation protein	TrbG [<i>Aromatoleum aromaticum</i> EbN1, plasmid 2] YP_195567	82
9	<i>trbH</i>	7767	8234	156	+	66	mating pair formation protein	TrbH [Uncultured bacterium, pTB11] YP_112375	57
10	<i>trbI</i>	8240	9634	465	+	63	mating pair formation protein	TrbI [Uncultured bacterium, pKJK5] YP_709150	69
11	<i>trbJ</i>	9653	10426	258	+	57	conjugation entry exclusion protein	TrbJ [<i>Achromobacter xylosoxidans</i> plasmid pA81] YP_195838	77
12	<i>trbK</i>	10437	10676	80	+	60	conjugation entry exclusion protein	TrbK [Uncultured bacterium, pB10] NP_857986	74
13	<i>trbL</i>	10681	12423	581	+	65	mating pair formation protein	TrbL [Uncultured bacterium, pB3] YP_133919	76
14	<i>trbM</i>	12443	13036	198	+	64	mating pair formation protein	TrbM [Uncultured bacterium, pB4] NP_598154	77
15	<i>trbN</i>	13050	13655	202	+	63	mating pair formation protein, muramidase/lysozyme	TrbN [<i>Bordetella pertussis</i> , pBP136] YP_787950	88
16	<i>trbO</i>	13681	13944	88	+	61	mating pair formation protein	Hypothetical protein [Acidovorax sp. JS42, pAOVO02] YP_974095	58
17	<i>trbP</i>	13947	14645	233	+	66	mating pair formation protein	TraX [<i>Acidovorax</i> sp. JS42,pAOVO02]	72

CDS#	Gene name	Start	Stop	Size of protein (aa)	Strand	GC(%)	Product	Most closely related gene product and its Genbank accession number	% aa Identity
18	<i>orf18</i>	15088	15573	162	+	67	protein	YP_974094	
							conserved hypothetical protein	Hypothetical protein [Collimonas fungivorans, pTER331] YP_001672028	62
19	<i>orf19</i>	15570	15977	136	+	61	conserved hypothetical protein	Hypothetical protein Phap_4422 [Polaromonas naphthalenivorans CJ2, pPNAP01] YP_973442	45
20	<i>parA</i>	15934	16305	124	-	69	resolvase	ParA [Uncultured bacterium, pB3] YP_133926	82
21	<i>merR</i>	16418	16852	145	-	61	regulatory protein	MerR [Delftia acidovorans] AAP88278	98
22	<i>merT</i>	16924	17274	117	+	62	mercuric ion transport protein	MerT [Uncultured bacterium, pSB102] NP_361069	97
23	<i>merP</i>	17287	17562	92	+	60	periplasmic mercuric ion binding protein	MerP [Pseudomonas aeruginosa, pVS6] ACA09394	98
24	<i>merC</i>	17590	18012	141	+	62	mercuric ion transport protein	MerC [Uncultured bacterium] BAH90196	93
25	<i>merA</i>	18052	19737	562	+	66	mercuric ion reductase	MerA [Delftia acidovorans] AAP88282	90
26	<i>merD</i>	19755	20120	122	+	69	regulatory protein	MerD [Pseudomonas aeruginosa, pVS6] ACA09397	98
27	<i>merE</i>	20117	20353	79	+	64	mercuric ion binding membrane protein	MerE [Halothiobacillus neapolitanus c2] ZP_03739630	98
28	<i>urf2</i>	20350	21342	331	+	64	conserved hypothetical protein	Urf2 [Pseudomonas sp. A19-1] CAD83842	86
29	<i>tniR</i>	21350	21964	205	-	65	resolvase	TniR [Uncultured bacterium, pTP6] YP_447046	94
30	<i>tniQ</i>	22025	23242	406	-	61	transposase	TniQ [Uncultured bacterium, pTP6] YP_447047	96
31	<i>tniB</i>	23239	24147	303	-	65	ATP binding protein	TniB [Pseudomonas aeruginosa, pBS228] YP_758692	99
32	<i>tniA</i>	24150	25829	560	-	64	transposase	TniA [Pseudomonas aeruginosa, pBS228] YP_758693	99
33	<i>traC</i>	26238	30413	1392	-	65	conjugative transfer protein, DNA primase	TraC2 [Achromobacter denitrificans, pEST4011] NP_990923	100
34	<i>traD</i>	30417	30794	126	-	69	conjugative transfer protein	TraD [Achromobacter denitrificans, pEST4011] NP_990925	100
35	<i>traE</i>	30799	32991	731	-	62	conjugative transfer protein, DNA topoisomerase	TraE [Achromobacter denitrificans, pEST4011] NP_990926	99
36	<i>traF</i>	33000	33536	179	-	63	mating pair formation, signal peptidase	TraF [Achromobacter denitrificans, pEST4011] NP_990927	100

CDS#	Gene name	Start	Stop	Size of protein (aa)	Strand	GC(%)	Product	Most closely related gene product and its Genbank accession number	% aa Identity
37	<i>traG</i>	33533	35446	638	-	63	coupling protein, Dtr/Mpt connection	TraG [Achromobacter denitrificans, pEST4011] NP_990928	99
38	<i>traH</i>	35742	36074	111	-	63	conjugative transfer protein, relaxosome stabilization	Hypothetical protein [Achromobacter denitrificans, pEST4011] NP_990930	97
39	<i>traI</i>	35443	37641	733	-	63	conjugative transfer protein, DNA relaxase	TraI [Achromobacter denitrificans, pEST4011] NP_990929	94
40	<i>traJ</i>	37676	38065	130	-	61	conjugative transfer protein, relaxosome component	TraJ [Achromobacter denitrificans, pEST4011] NP_990932	98
41	<i>traK</i>	38468	38868	134	+	59	conjugative transfer protein, relaxosome component	TraK [Achromobacter denitrificans, pEST4011] NP_990933	99
42	<i>traL</i>	38869	39594	242	+	59	conjugative transfer protein	TraL [Achromobacter denitrificans, pEST4011] NP_990934	100
43	<i>traM</i>	39591	40031	146	+	64	conjugative transfer protein	TraM [Achromobacter denitrificans, pEST4011] NP_990935	100
44	<i>kfrC</i>	40101	40751	217	-	65	stable inheritance protein	Hypothetical protein [Achromobacter denitrificans, pEST4011] NP_990936	93
45	<i>kfrB</i>	40782	41126	115	-	54	stable inheritance protein	Hypothetical protein [Achromobacter denitrificans, pEST4011] NP_990876	97
46	<i>orf46</i>	41143	41753	204	-	58	conserved hypothetical protein	Hypothetical protein [Achromobacter denitrificans, pEST4011] NP_990877	100
47	<i>orf47</i>	41750	41938	63	-	67	conserved hypothetical protein	Hypothetical protein [Achromobacter denitrificans, pEST4011] NP_990878	100
48	<i>kfrA</i>	42003	43019	339	-	70	stable inheritance protein	KfrA [Achromobacter denitrificans, pEST4011] NP_990879	99
49	<i>korB</i>	43211	44284	358	-	64	regulation and partitioning protein	KorB [Achromobacter denitrificans, pEST4011] NP_990880	100
50	<i>incC</i>	44281	45372	364	-	62	partitioning protein	IncC1 [Achromobacter denitrificans, pEST4011] NP_990881	96
51	<i>korA</i>	45051	45359	103	-	60	transcriptional repressor protein	KorA [Achromobacter denitrificans, pEST4011] NP_990883	94
52	<i>kleF</i>	45522	45779	86	-	64	stable inheritance protein	KleF [Achromobacter denitrificans, pEST4011] NP_990884	97
53	<i>kleE</i>	45781	46227	149	-	61	stable inheritance protein	KleE [Achromobacter denitrificans, pEST4011] NP_990885	97
54	<i>kleA</i>	46221	46478	86	-	60	stable inheritance protein	KleA [Achromobacter denitrificans, pEST4011] NP_990886	100
55	<i>korC</i>	46669	46926	86	-	64	regulatory protein	KorC [Achromobacter denitrificans, pEST4011] NP_990887	100

CDS#	Gene name	Start	Stop	Size of protein (aa)	Strand	GC(%)	Product	Most closely related gene product and its Genbank accession number	% aa Identity
56	<i>kicB</i>	46941	48347	469	-	66	stable inheritance protein	pEST4011] NP_990887 KicB [<i>Achromobacter denitrificans</i> , pEST4011] NP_990888	99
57	<i>kicA</i>	48395	48829	145	-	63	antirestriction protein	KicA [<i>Achromobacter denitrificans</i> , pEST4011] NP_990889	100
58	<i>orf58</i>	48927	49211	95	-	64	conserved hypothetical protein	Orf10 [<i>Burkholderia cepacia</i> , pIJB1] AAZ08206	98
59	<i>tnpA</i>	50432	53461	1010	-	63	Tn3 family transposase	Transposase [<i>Pseudomonas aeruginosa</i> PA7] YP_001350671	100
60	<i>tnpR</i>	53458	54051	198	-	66	Tn3 family resolvase	Resolvase, N-terminal domain [<i>Ralstonia pickettii</i> 12] YP_001899048	100
61	<i>orf61</i>	54290	54568	93	+	63	putative antitoxin protein	Hypothetical protein [Uncultured bacterium, pB8] YP_358814	100
62	<i>relE</i>	54565	54849	95	+	62	toxin protein	ParE [Uncultured bacterium, pB8] YP_358815	98
63	<i>orf63</i>	55445	55816	124	+	53	conserved hypothetical protein	Hypothetical protein Rmet_6340 [<i>Ralstonia metallidurans</i> CH34] YP_582149	100