The complete sequences of plasmids pB2 and pB3 provide evidence for a recent ancestor of the IncP-1/β group without any accessory genes

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The nucleotide sequences of the broad-host-range antibiotic resistance plasmids pB2 (61 kb) and pB3 (56 kb), which were isolated from a wastewater treatment plant, were determined and analysed. Both have a nearly identical IncP-1/β backbone, which diverged early from the sequenced IncP-1/β plasmids R751, pB10, pJP4, pADP1 and pUO1. In contrast to the latter plasmids, the pB2 and pB3 backbone does not seem to have undergone any deletions. The complete partition gene parA is located downstream of the mating pair formation (trb) module. A 14-4 kb or 19-0 kb mobile genetic element is present between traC and parA of pB3 and pB2, respectively. This region is typical for insertions in IncP-1/β plasmids, but the insertion site is unique. Both elements differ only by a duplication in pB2 of a tetA–tetR–tnpA26 fragment. The 5 bp target site duplication and the 26 bp inverted repeats flanking the mobile genetic elements are still intact, indicating that the insertion occurred recently. The element consists of three nested transposable elements: (i) a relict of a Tn402-like transposon with a gene for a new class D β-lactamase (bla<sub>NPS-2</sub>); (ii) within that, another Tn402-like element with a class 1 integron harbouring the gene cassettes cmlA1 for a chloramphenicol efflux protein and adaA2 encoding a streptomycin/spectinomycin adenylyltransferase, and a copy of IS6100; (iii) into the integrase gene intI1 a tetracycline resistance module tetA–tetR flanked by copies of IS26 is inserted. Interestingly, in contrast to all other IncP-1/β plasmids analysed so far, the oriV region between trtA and klcA is not interrupted by accessory genes, and there is no indication that previously inserted accessory genes have subsequently been deleted. The genes kluAB are also missing in that region and should thus be considered acquired genes. These findings, together with the fact that IncP-1/β plasmids acquired accessory elements at various positions in the backbone, suggest that IncP-1/β plasmids without any accessory genes exist in microbial communities. They must occasionally acquire accessory genes by transposition events, resulting in those plasmids that have been found based on selectable phenotypic traits.

INTRODUCTION

Horizontal gene transfer mediated by plasmids plays an important role in the evolution and adaptability of bacteria (Davison, 1999). Plasmids of the incompatibility group IncP-1 are of particular interest because they are able to self-transfer and be stably maintained in a wide range of Gram-negative bacteria (Thomas & Smith, 1987). Their conjugation system even promotes transfer to Gram-positive bacteria and yeasts (Trieu-Cuot et al., 1987; Sikorski et al., 1990). Given their promiscuity and high prevalence in the environment, they serve as important vectors for the horizontal mobility of accessory genes that code for degradation of pollutants, resistance to antibiotics or heavy metals, and so far unknown functions (Top et al., 2000; Heuer et al., 2002; Dröge et al., 2000). Due to the particular interest in IncP-1 plasmids, they are one of the most intensively studied groups, and seven naturally occurring members of the group have already been completely sequenced: RP4, representing a number of indistinguishable IncP-1α antibiotic resistance plasmids (Pansegrau et al., 1994), and members of the phylogenetically distinct IncP-1β group (Thomas & Smith, 1987), namely R751 (Thorsted et al., 1998), pB4 (Tauch et al., 2003b), pB10 (Schlüter et al., 2003) (all encoding multiple antibiotic resistances), pADP1...
(Martinez et al., 2001), pUO1 (Sota et al., 2003) and pJP4 (Trefault et al., 2004) (encoding degradation of herbicides and haloacetates). The phylogeny of this diverse IncP-1 group is, however, not yet well understood.

Even though IncP-1 plasmids are infectiously transmitted with high rates, it has been assumed that horizontal transfer is not sufficient for plasmids to be maintained as genetic parasites, given the burden they present to their host, so that they need to carry at least intermittently advantageous traits to be maintained in bacterial populations (Bergstrom et al., 2000). This hypothesis is supported by the fact that so-called ‘cryptic’ plasmids of the IncP-1 group, comprised solely of genes for replication, stable inheritance, and horizontal transfer, have not been found so far. All known IncP-1 plasmids have, instead, large regions with acquired genes encoding various resistance or degradation traits, which occasionally might augment the fitness of the bacterial host. One of the insertion regions is located near the origin of replication oriV, separating it from the replication gene trfA (Smith et al., 1993). The other insertion regions are downstream of traC and, in pB4 only, downstream of upf54.4. Here we present the first complete sequence of two promiscuous IncP-1β plasmids that have no insertions in the oriV region and no remnants of deleted transposable elements. Presumably they have recently evolved from a ‘cryptic’ ancestor. Sequence comparison with the other sequenced IncP-1β plasmids shows evidence that R751, pB10, pJP4, pADP1 and pUO1 also descended from such a cryptic ancestor.

METHODS

Bacterial strains and growth conditions. Escherichia coli DH5α mcr (Grant et al., 1990) containing the multiresistance plasmid pB2 or pB3 was grown at 37°C in Luria Broth (LB) medium supplemented as needed with antibiotics at the following final concentrations: streptomycin, 40 μg ml⁻¹, or tetracycline, 5 μg ml⁻¹. For solid media, 15 g agar per litre medium was added. Indicator medium for strains expressing an active β-galactosidase was supplemented with 40 μg X-Gal ml⁻¹ (final concentration).

Standard DNA techniques. Plasmid DNA from the plasmid-containing E. coli DH5α mcr derivatives was extracted with the Nucleobond Kit PC100 on AX100 columns (Macherey-Nagel) according to the protocol supplied by the manufacturer. Recombinant plUC19 derivatives were isolated using the QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer’s instructions. DNA was extracted from agarose gels with the Sephaglas BandPrep Miniprep Kit (Qiagen) and purified on Sephacryl MicroSpin S-400 HR columns (Amersham Pharmacia Biotech). Restriction enzyme digestion, agarose gel electrophoresis, DNA cloning and transformation of E. coli DH5α mcr was carried out according to Sambrook et al. (1989).

Construction of a shotgun library and DNA sequencing of pB2 and pB3. Purified plasmid DNA was randomly fragmented and the 1-0-1.5 kb size fraction was cloned into the sequencing vector pUC19 (Qiagen). Plasmid DNA was prepared from E. coli DH10B shotgun clones with the BioRobot 9600 (Qiagen). Standard shotgun sequencing reactions using the dye-terminator were separated on an ABI 3700 (Applied Biosystems) DNA sequencer, resulting in 825 and 892 sequencing reads with 589 and 578 bases mean sequence length for pB2 and pB3, respectively. Sequencing reads were assembled using the phred/phrap assembly (Ewing et al., 1998). Gap closure and polishing of the sequence was done by primer walking using the dye-terminator chemistry on an ABI 377 sequencer (IIT Biotech). A 4-6 kb EcoRI restriction fragment of pB2 was cloned and sequenced by primer walking to verify the duplication of tetA(C)–tetR-IS26.

RESULTS AND DISCUSSION

Complete sequences of the environmental plasmids pB2 and pB3

The antibiotic resistance plasmids pB2 and pB3 were captured by exogenous isolation in Pseudomonas sp. GFP1 from a wastewater treatment plant in Germany, as were the previously described IncP-1 plasmids pB4 and pB10 (Dröge et al., 2000). They mediate resistance against the antimicrobial agents streptomycin, spectinomycin, ampicillin, sulfonamides, chloramphenicol and tetracycline. Complete sequencing of both plasmids with an approximately eight-fold coverage resulted in circularly closed sequences of 56 167 bp for pB3 and 60 732 bp for pB2, with a mean G + C content of 63.8 % and 63.3 %, respectively. It thus appears that restriction analysis had underestimated the sizes of these plasmids (Dröge et al., 2000). The sequences of pB2 and pB3 were identical, except for 1 base pair in the oriV region (T replaced by C in pB2 at position 423) and a duplication in pB2 of the 4565 bp tetA–tetR–tnpA526 fragment (see below). Plasmid pB3 may thus be interpreted as a pB2 deletion derivative, which could have arisen by homologous recombination via directly repeated IS26 elements. The NotI restriction patterns and antibiotic resistances predicted from the sequences of both plasmids corresponded to the findings of Dröge et al. (2000) (data not shown), but the reported low conjugative transfer rates of pB3 were not reproducible. Using exactly the same strains, protocols and laboratory (University of Bielefeld) as in the study of Dröge et al. (2000), the plasmid transfer frequencies were nearly identical for both plasmids, and comparable to those reported previously for pB2. When the transferability of both plasmids was tested with different strains, using a slightly different filter mating protocol (Top et al., 1992) in a different laboratory (University of Idaho), both plasmids transferred at equally high rates of more than 0.1 per recipient between E. coli strains DH5α and K12Rif (a rifampicin-resistant derivative of MG1655) and between E. coli DH5α and Pseudomonas putida UWC6. Both plasmids transferred at frequencies of approximately 10⁻³ per
recipient from *E. coli* DH5α to *Sinorhizobium meliloti* Rm1021 (data not shown) under the same conditions. This lack of difference in transferability between the two plasmids is in agreement with their very high sequence similarity. In the following, only the sequence of pB3 will be discussed.

Sequence annotation of pB3 revealed the typical structure of an IncP-1β plasmid backbone (Fig. 1), as described for R751 (Thorsted *et al*., 1998): the regions Tra1 (*tra*) and Tra2 (*trb*) with genes required for conjugative transfer and the origin of conjugative replication *oriT*, the central control region (Ctl) harbouring genes for regulation and stable inheritance, the replication genes *trfA* and *ssb*, and the origin of vegetative replication *oriV*. The partitioning gene *parA* is located downstream of *upf31.0*. The encoded *parA* gene product is homologous to ParA of the IncP-1β plasmid pB4 (Tauch *et al*., 2003) (94% identity) and to ParA of the IncP-1α plasmid RP4 (Pansegrau *et al*., 1994) (75% identity), belongs to the PinR family of site-specific recombinases (COG1961) and possesses the N-terminal domain of resolvases (Pfam00239). Plasmid pB3 contains an intact *parA* gene, whereas the 5’ part of *parA* has been deleted in pB4 and R751 (Thorsted *et al*., 1998), and the entire gene is absent in the IncP-1β plasmids pB10 (Schlüter *et al*., 2003), pUO1 (Sota *et al*., 2003), pJP4 (Trefault *et al*., 2004) and pADP1 (Martinez *et al*., 2001). This ParA protein encoded on pB3 might enhance stable inheritance of the plasmid by resolution of multimers.

The backbone of plasmid pB3 is only interrupted once by an insertion of a composite mobile genetic element, between Tra1 and Tra2. This acquired 14-4 kb mobile genetic element contains genes mediating antibiotic resistances and transposition. The pB3 region downstream of the replication initiation gene *trfA* does not contain any accessory genes, and neither relics nor footprints of transposons similar to Tn3, Tn21 or Tn501 could be found. To our knowledge, this is the first example of an IncP-1 plasmid with a contiguous *oriV* region between *trfA* and *klcA*.

![Fig. 1. Genetic map of the IncP-1β plasmid pB3. Coding regions are shown by arrows indicating the direction of transcription. The regions *Tra*1 (*tra*), *Tra*2 (*trb*), the replication (*rep*) module (*trfA–ssb*), the central control region encoding regulatory and stability functions (Ctl, *klcA–upf54*–4), and the partition gene *parA* are differentiated by colour. The complex 14-4 kb ‘genetic load’ region downstream of the conjugative transfer gene *traC* consists of different distinct mobile genetic elements: i) a Tn402 relict carrying the β-lactamase gene *bla*<sub>NPS-2</sub> and the resolvase gene *tniC*, ii) a composite tetracycline (*tet*) resistance transposon with IS26 elements (light green), iii) another Tn402 derivative integrating a class 1 integron (lilac) and an IS6100 copy. The locations of the origins of vegetative (*oriV*) and transfer (*oriT*) replication are indicated by black circles.](http://mic.sgmjournals.org)
Plasmid pB3 diverged early from IncP-1β plasmids similar to R751

The availability of seven complete sequences of IncP-1 plasmids provides a large dataset to infer a phylogeny of this plasmid group. Maximum-likelihood trees of different parts of the backbone were constructed in order to see whether they show a common phylogeny or whether recombination events affect the evolutionary histories. The phylogenetic trees calculated on the basis of the conserved IncP-1 backbone regions Tra1 (traC–traM, 13-8 kb in pB3), Tra2 (trba–trbp, 13-6 kb in pB3) and trfA–ssb (1-6 kb in pB3) have basically identical topologies (Fig. 2). Plasmid RP4 represents the distant IncP-1α group, and the IncP-1β plasmid pB4 diverged early from pB3 and the other four plasmids, which form the ‘R751 group’ (plasmids R751, pUO1, pADP1 and pB10). R751 is the best-studied IncP-1β plasmid, and the other three plasmid backbones show on average more than 95% identity to R751. Several other resistance and degradative plasmids belong to this group as well, such as R906, R772 and pTSA (Smith & Thomas, 1987; Schlüter et al., 2003; Tralau et al., 2001). Plasmid pB3 is clearly separated from this R751 group in these three backbone regions. The tree of the Ctl region (klcA–upf54–4, 8-3 kb in pB3) seems to be different, because pB10 appears to be more related to pB4. This is due to the recently described recombination of part of the central control region between incC2 and oriV of pB10 with a pB4-like plasmid (Schluter et al., 2003). This is made clear by the change in tree topology of the adjacent genes korB and incC2. The tree of korB (and kfrA, data not shown) supports the phylogeny inferred from the other backbone regions, while in the tree of incC2 (and klcA, data not shown) pB10 clusters with pB4. The same is true for the recently sequenced catabolic plasmid pJP4 (Trefault et al., 2004), as its backbone is nearly identical to that of pB10 (only 5 base changes in 39 030 bp, without considering columns with gaps in the alignment). In conclusion, the plasmid pB3, which lacks an insertion in the oriV region, and the R751 group of plasmids (R751, pUO1, pADP1, pJP4 and pB10), which all have transposable elements inserted in proximity to oriV, seem to be derived from a common ancestor.

Insertion regions of accessory elements in pB3 and other IncP-1 plasmids

IncP-1 plasmids generally acquire mobile genetic elements in the regions between the Tra1, Tra2 and Ctl modules, i.e. downstream of traC, trfA and upf54.4. Only pB4 is known so far to have an accessory element inserted in the upf54.4 region, while pB3, described here, is the first plasmid reported only to harbour an insertion downstream of traC. The insertion regions of IncP-1β plasmids all have in common Rho-independent transcriptional terminators and palindromic repeats of unknown function. These repeats of R751, pB10, pJP4, pADP1 and pB4 have the consensus sequence CATCGcCAtttctGaCGatG (capitals symbolize conserved bases), and are often associated with restriction enzyme target sites. The 16 repeats of pB3 have a similar consensus sequence, CATCGcCAttctGaCGatG. It has been hypothesized that these repeats play a role in directing insertions (Thorsted et al., 1998).

The borders of acquired genetic elements in the completely sequenced IncP-1 plasmids were identified based on similarities to transposable elements, inverted repeats, target-site duplications and adjacent sequences common to other IncP-1 plasmids. The remaining backbone sequences were aligned to infer the structure of a common ancestor in the insertion regions. The 1·5 kb oriV region between trfA and klcA of pB3 is contiguous, as predicted for an ancestral IncP-1 replication system, but unlike all known naturally occurring IncP-1 plasmids (Smith et al., 1993). It harbours all the typical sequence motifs of protein binding sites:

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**Fig. 2.** Phylogeny of the IncP-1 backbone regions Tra1 (traC–traM), Tra2 (trba–trbp), the replication region (trfA–ssb) and the central control region (klcA–upf54–4). The recombination of part of the central control region between incC2 and oriV of pB10 with a pB4-like plasmid is shown by the change in tree topology from korB to incC2. Maximum-likelihood trees (DNAmlk 3.5 by J. Felsenstein) were based on sequence alignments (CLUSTAL W), where columns with gaps were removed.
A/T- and G/C-rich regions of the oriV, palindromic repeats and the transcriptional terminator downstream of trfA (Fig. 3a). The last two features are missing in RP4. Plasmids pB10, pJP4 and pB4 only have two TrfA-binding sites left of the DnaA-binding site, as shown in Fig. 3a. In fact, this region (Fig. 3a, up to the red bracket in pB10) seems to be the left-hand end of the fragment in pB10, which presumably entered by homologous recombination with a pB4-like plasmid (Schlueter et al., 2003). Both plasmids pB10 and pJP4 share a recombined region similar to pB4 between oriV and incC2, and their backbone sequences in the oriV region are identical, except for a 388 bp deletion adjacent to the insertion site in pJP4. In pUO1 and pADP1, deletions between short direct repeats have removed the fourth palindromic repeat. In plasmid pB3, there is no evidence for the deletion of an accessory element which might have previously been inserted in the oriV region. All the other plasmids have mobile genetic elements, or remnants of them, inserted at various positions. Thus, in this region, each of these plasmids appears to originate from an ancestor like pB3.

Upstream of klcA, between the binding sites for KorB and TrfA, the hypothetical genes with unknown functions kluA and kluB were found in R751 and pADP1, but not in any of the other plasmids (Fig. 3b). The kluA gene has the SD motif GGAGG, representing a strong ribosome-binding site. It presumably encodes a protein of the COG3609 group (predicted transcriptional regulators containing the CopG/Acr/MetJ DNA-binding domain). The kluB gene is transcriptionally linked to kluA, and also has a strong SD sequence (GAAGG). It may code for a ParE-like plasmid-stabilization protein of the groups COG3668 and Pfam05016. If pB3 indeed resembles the ancestral sequence in the oriV region, then kluA and kluB must be acquired genes. These genes are missing not only in pB3 but also in the plasmids pB4, pB10, pJP4 and pUO1. Instead, pB4, pB10 and pJP4 carry different insertions, with a predicted DNA-damage-inducible (din) gene adjacent to, and the promoter region of din overlapping with, the KorB-binding motif. A 17 bp fragment adjacent to this motif is common to pB3, R751 and pADP1, but is not present in pUO1, while pUO1 has a fragment (presumably a relict) of the hypothetical kluB gene in common with R751 and pADP1, but not with pB3. It might be speculated that, in the pB4-lineage, a fragment carrying the din gene was inserted, which was later horizontally transferred to an ancestor of pB10 and pJP4; in the R751–lineage, a fragment with kluAB was
inserted and kluB became an ORF with the TGA stop codon next to the insertion. A fragment adjacent to the KorB-binding motif was then deleted from pUO1.

The only acquired mobile genetic element in pB3 was found between the transcriptional terminator and the palindromic repeats downstream of traC and upstream of parA, i.e. between the trb and the tra region (Fig. 4). The 5 bp target site duplication of the insertion and the 26 bp inverted repeats of the mobile genetic element are still intact (Fig. 4, lower part), indicating that the insertion occurred recently into an ancestral plasmid without any accessory genes. The insertion site is identical to that of the Tn402-like mer transposon Tn4672 in pUO1. The mobile genetic elements of R751, pADP1 and the plasmids from the same wastewater plant, pB10 and pB4, have different insertion sites downstream of traC (Fig. 4). Interestingly, plasmid pJP4 has no insertion downstream of traC, and its backbone is completely homologous to that of pB3 in this region. The inverted repeats flanking the composite mobile genetic element in pB3 differ from those in R751 and pUO1 by two mismatches (Fig. 4, lower part). In all plasmids analysed, except pB3, the left-hand inverted repeats and part of the backbone to the left of the insertion sites were deleted to various extents. In contrast, the backbone to the right of the insertion sites remained intact. The genetic mechanism responsible for such asymmetric deletions is not clear.

The findings described above strongly suggest that all the plasmids of the IncP-1β group descended from a ‘cryptic’ ancestor, as they have different acquired genetic elements inserted at various positions, and remnants of deleted common insertions are missing. Thus, IncP-1 plasmids without any accessory genes, which have occasionally been modified by transposition events, probably exist in microbial communities. They have remained undiscovered because they do not confer the phenotypic traits that researchers are selecting for when screening for the presence of plasmids. The presence of cryptic IncP-1 plasmids over long evolutionary times seems to contradict model predictions that they need to confer at least intermittently advantageous traits to be maintained in bacterial populations, given the burden they present to their host (Bergstrom et al., 2000). The existence of cryptic IncP-1 plasmids might be explained by a low metabolic cost due to the tight transcriptional and translational control of gene expression of backbone genes (Bingle et al., 2003; Zatyka et al., 1997) and a low copy number. Without accessory genes, the burden of the plasmid to the host is even lower, and might well be balanced by a high rate of infectious transfer such as that observed for pB3 and pB2. Moreover, it has been shown that some plasmids can abolish their burden by coevolution with their host (Bouma & Lenski, 1988), or even confer a yet-unexplained fitness advantage in a naive host (Enne et al., 2004). Some backbone genes of the plasmid might also confer advantages to the host, by promoting biofilm formation for example (Ghigo, 2001).

The pB3 ‘genetic load’ downstream of the conjugative transfer gene traC contains three distinct mobile genetic elements

A 14.4 kb mobile genetic element flanked by the 5 bp target duplication and the 26 bp inverted repeats is inserted between traC and parA (Fig. 5). It contains a gene for a new class D β-lactamase (bla₈PS-2), a tetracycline resistance module tetA(C)–terR, which is flanked by copies of IS26, and a class 1 integron terminated by an insertion of IS6100. The bla₈PS-2 gene upstream of parA encodes a β-lactamase that is, respectively, 72 % and 71 % identical to the LCR-1 β-lactamase of Pseudomonas aeruginosa (Couture et al., 1992) and the NPS-1 β-lactamase of plasmid pB4 from the same wastewater treatment plant (Tauch et al., 2003b; Pai & Jacoby, 2001), and thus represents a new variant of this

Fig. 4. Comparison of the region between trb and traC of pB3 with other completely sequenced IncP-1β plasmids. MGE, mobile genetic element insertion; t, Rho-independent transcriptional terminator downstream of traC; R, palindromic repeats. Lower panels: inverted repeats and adjacent sequences, showing the 5 bp target site duplication of pB3 and distinct insertion sites for pB10 (identical site in pJP4), pB3 and R751.

![Image](https://example.com/image.png)
enzyme type. The NPS-2 β-lactamase belongs to class D (COG2602), which also includes LCR-1 of *P. aeruginosa* and NPS-1 encoded by pB4 (Tauch *et al.*, 2003b). The resolvase gene *tniC* located downstream of *bla*<sub>NPS-2</sub> is 98 % identical to *tniC* of transposon Tn402/Tn5090 on R751 (Radström *et al.*, 1994). As described for ParA, the *tniC* gene product also is a member of the site-specific recombinase family (COG1961 and Pfam00239). The *tniC* gene might be regarded as a relict of the Tn402/Tn5090-specific transposition module, which originally consisted of the genes *tniC*, *tniQ*, *tniB* and *tniA* (Radström *et al.*, 1994). The organization of the pB3 *parA–bla*<sub>NPS-2–tniC</sub> segment is reminiscent of the corresponding region on plasmid pB4, with the difference that pB4 *parA* has been deleted and the *bla*<sub>NPS-1</sub> gene has inserted downstream of *tniC* on pB4. Apparently, the *parA–tniC* intergenic region contains a preferred site for insertion of cassette-like elements containing β-lactamase genes.

Another mobile genetic element with similarity to Tn402 integrated upstream of *tniC* on pB3. This composite transposable element carries the tetracycline-resistance module *tetA(C)–tetR(C)*, which is flanked by copies of the insertion sequence IS26. Insertion of the composite tetracycline (*tet*) transposon caused an 8 bp target-site duplication (TTCTACGG). The tetracycline efflux protein TetA(C) is identical to the corresponding protein of the *Aeromonas salmonicida* plasmid pRAS3 (L’Abee-Lund & Sorum, 2002), whereas the tetracycline repressor TetR(C) is identical to TetR of the *Salmonella typhimurium* IncN plasmid R46 (NP_511232). Previously, Schnabel & Jones (1999) sequenced the transposon Tn1404 carrying IS26–*tetR–tetC–IS26* of *Pseudomonas* sp. R9, which was isolated from a Michigan apple orchard. Available sequence data for the tetracycline transposon, including its insertion site and adjacent integron-specific sequences, are identical to those of the corresponding pB3 segment. These findings lead to the assumption that the pB3 host bacterium and *Pseudomonas* sp. R9 have captured a transposable integron derivative integrating a tetracycline resistance transposon from a common ancestor. Unfortunately, the tetracycline resistance genes of *Pseudomonas* sp. R9 Tn1404 have not been sequenced.

The class 1 integron on pB3 possesses two resistance gene cassettes: *cmlA1* for a chloramphenicol efflux protein and *aadA2* encoding a streptomycin/spectinomycin adenylyltransferase. The integron-specific 3′ segment consists of the genes *qacEΔ1–sul1–orf5–orf6* and *orf6* (conserved hypothetical protein), and is terminated by the insertion of IS6100. IS6100 is flanked by an additional internal copy of the class 1 integron outer end (123 bp IRT end), whereas, on the opposite side, only the 25 bp IRT motif is present. The pB3 integron 3′-segment variant including a terminal IS6100 copy has been frequently found and has been described in detail for the R plasmids pCG4 and pTET3 of *Corynebacterium glutamicum* (Tauch *et al.*, 2002, 2003a), the IncU R plasmid pRAS1 of the fish pathogen *Aeromonas salmonicida* (Sorum *et al.*, 2003),
Tn2521–In33 inserted in the chromosome of *P. aeruginosa* Dalgleish (Partridge *et al*., 2002), plasmid R1033 Tn1696–In4 of *P. aeruginosa* (Partridge *et al*., 2001), the multidrug resistance genomic island 1 of *Salmonella enterica* serovar Typhimurium (Boyd *et al*., 2001) and the mobile genome island pKLC102 of *P. aeruginosa* C (Klockgether *et al*., 2004), demonstrating the wide distribution of this integron derivative. Although the pB3 integron does not possess a functional transposition module, the element most probably entered the plasmid by transposition into the resolution site upstream of the resolvase gene *tniC*. The integron insertion site is flanked by 5 bp direct repeats representing the target-site duplication (GGACT). The *tniC* upstream regions including the integron target site are almost identical on pB3 and pB4.

In summary, the complex 14·4 kb ‘genetic load’ region downstream of *traC* contains five different antibiotic resistance genes, conferring resistance to ampicillin, tetracycline, chloramphenicol, spectinomycin, streptomycin and sulfonamides, which is in agreement with the observed resistance spectrum of *E. coli* DH5α carrying pB3. The resistance genes are organized on gene cassettes or transposable elements.

Conclusions

Plasmid pB3 and the five sequenced plasmids of the R751 group have a common ancestor. All these plasmids acquired genetic elements in different positions of the oriV region and downstream of *traC*, with the exception of plasmids pB3 and pJP4, which lack an acquired genetic element in one or other of these two regions. The most straightforward explanation of the evolutionary history of these plasmids is the existence of a common ancestor free of acquired genetic elements. The alternative explanation, that acquired elements were deleted without trace, for example by recombination with another IncP-1β plasmid, and replaced by other elements at a different position each time one of the plasmids evolved, seems to be less likely. Therefore, IncP-1β plasmids without any accessory genes probably exist in microbial communities and occasionally acquire accessory genes by transposition events, resulting in those plasmids that have been found today based on selectable phenotypic traits. Methods such as triparental exogenous plasmid isolation may be a very good approach to capture such IncP-1 plasmids from various habitats, since the selection is based solely on the ability of the plasmid to mobilize a non-conjugative vector, and not on any phenotypic trait (Hill *et al*., 1992; Top *et al*., 1994). The use of antibiotics selects for plasmids like pB3, which serve as vectors for the spread of accumulated resistance genes between species and habitats, temporarily providing their hosts with a competitive advantage. Similarly, the presence of recalcitrant xenobiotic compounds, such as atrazine and 2,4-D, selects for those same plasmid ancestors to acquire the operons required for degradation of these molecules, thus giving their hosts access to an additional carbon and/or nitrogen source. However, the mobile and promiscuous IncP-1 plasmid backbones alone seem to provide sufficient benefits to a bacterial community for them to be maintained over longer evolutionary times.

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