

The 64 508 bp IncP-1 β antibiotic multiresistance plasmid pB10 isolated from a waste-water treatment plant provides evidence for recombination between members of different branches of the IncP-1 β group

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The complete 64 508 bp nucleotide sequence of the IncP-1 β antibiotic-resistance plasmid pB10, which was isolated from a waste-water treatment plant in Germany and mediates resistance against the antimicrobial agents amoxicillin, streptomycin, sulfonamides and tetracycline and against mercury ions, was determined and analysed. A typical class 1 integron with completely conserved 5' and 3' segments is inserted between the *tra* and *trb* regions. The two mobile gene cassettes of this integron encode a β -lactamase of the oxacillin-hydrolysing type (Oxa-2) and a gene product of unknown function (OrfE-like), respectively. The pB10-specific gene load present between the replication module (*trfA1*) and the origin of vegetative replication (*oriV*) is composed of four class II (Tn3 family) transposable elements: (i) a Tn501-like mercury-resistance (*mer*) transposon downstream of the *trfA1* gene, (ii) a truncated derivative of the widespread streptomycin-resistance transposon Tn5393c, (iii) the insertion sequence element IS1071 and (iv) a Tn1721-like transposon that contains the tetracycline-resistance genes *tetA* and *tetR*. A very similar Tn501-like *mer* transposon is present in the same target site of the IncP-1 β degradative plasmid pJP4 and the IncP-1 β resistance plasmid R906, suggesting that pB10, R906 and pJP4 are derivatives of a common ancestor. Interestingly, large parts of the predicted pB10 restriction map, except for the tetracycline-resistance determinant, are identical to that of R906. It thus appears that plasmid pB10 acquired as many as five resistance genes via three transposons and one integron, which it may rapidly spread among bacterial populations given its high promiscuity. Comparison of the pB10 backbone DNA sequences with those of other sequenced IncP-1 β plasmids reveals a mosaic structure. While the conjugative transfer modules (*trb* and *tra* regions) and the replication module are very closely related to the corresponding segments of the IncP-1 β resistance plasmid R751 and even more similar to the IncP-1 β degradative plasmids pTSA and pADP-1, the stable inheritance operons *klcAB-korC* and *kleAEF* are most similar to those of the IncP-1 β resistance plasmid pB4, and clearly less similar to the other IncP-1 β plasmids. This suggests that IncP-1 β plasmids can undergo recombination in the environment, which may enhance plasmid diversity and bacterial adaptability.

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INTRODUCTION

Mobile genetic elements, especially plasmids, and their horizontal transfer play an important role in the evolution and adaptability of prokaryotes (Davison, 1999). Plasmids have been studied extensively as vehicles that promote the dissemination of antibiotic-resistance determinants

(Davies, 1994; Mazel & Davies, 1999; Davison, 1999). More recent work has shown that they also facilitate the adaptation of bacteria to environments contaminated with toxic xenobiotics by exchanging genes and entire operons that code for the degradation of these pollutants (Smalla & Sobczyk, 2002; Top *et al.*, 2002). Detailed analyses of antibiotic-resistance plasmids isolated from clinical and environmental bacteria [reviewed by Davies (1994), Mazel & Davies (1999) and Davison (1999)] revealed that several of these plasmids belong to the IncP-1 incompatibility group and serve as vectors for the horizontal mobility of the encoded accessory genes (Smith & Thomas, 1987; Heuer *et al.*, 2002; van Overbeek *et al.*, 2002; Dröge *et al.*, 2000). IncP-1 plasmids are very promiscuous, as they are able to self-transfer and be stably maintained in a wide range of Gram-negative bacteria (Thomas & Smith, 1987). The prototype IncP-1 β resistance plasmid R751 contains the resistance genes *dhfrIIIc* encoding a dihydrofolate reductase for trimethoprim resistance and *qacE* encoding a small exporter protein mediating resistance to quaternary ammonium compounds and disinfectants. Two transposable elements, the cryptic Tn4321 and the integron-containing Tn402/5090 are inserted downstream of the replication gene *trfA1* and the conjugative transfer gene *traC* of R751, respectively (Thorsted *et al.*, 1998). Comparison of the R751 sequence with the one of the IncP-1 α R-plasmid RP4 (Pansegrau *et al.*, 1994) confirms the conservation of the IncP backbone modules for replication, stable inheritance functions and conjugative transfer between the two branches of this family (Thorsted *et al.*, 1998).

Several degradation pathways are also frequently found to be located on IncP-1 β plasmids (Top *et al.*, 2002). For example, plasmid pJP4 isolated from *Ralstonia eutropha* was found to be a member of the IncP-1 β subgroup. This plasmid contains genes (*tfd*) encoding enzymes involved in the catabolism of 2,4-dichlorophenoxyacetate and 3-chlorobenzoate (Don & Pemberton, 1985; Clément *et al.*, 2001). Two degradative IncP-1 β plasmids were respectively completely and almost completely analysed at the DNA sequence level: pTSA from *Comamonas testosteroni*, encoding the widespread genes for *p*-toluenesulfonate degradation (*tsa*) (Tralau *et al.*, 2001), and pADP-1 from *Pseudomonas* sp. strain ADP, which mediates the metabolism of the herbicide atrazine (Martinez *et al.*, 2001). These plasmids contain IncP-1 β -specific backbone modules for replication, stable inheritance and conjugative transfer which show high similarity to the corresponding modules of the IncP-1 β resistance plasmid R751. In between these backbone regions, putative transposable elements that carry the degradative genes are inserted.

It thus appears that the IncP backbone can either carry antibiotic-resistance determinants or degradative operons and that the encoded backbone functions facilitate the dissemination of these determinants between diverse bacterial species. So far as we know, a plasmid that carries both types

of genes (antibiotic-resistance and degradative genes) has not yet been identified.

It is generally accepted that insertion sequence (IS) elements and transposons play an important role in the modular evolution of plasmids, which was impressively documented for the multiresistance plasmid pTP10 isolated from the clinical isolate *Corynebacterium striatum* M82B. This plasmid represents a mosaic structure composed of DNA segments originating from soil bacteria and plant, animal and human pathogens (Tauch *et al.*, 2000). Mosaic structures of plasmids can also result from recombination between plasmids carrying homologous DNA segments. This was demonstrated for a natural population of *Escherichia coli* harbouring F-like plasmids. It could be shown that recombination between genes of these plasmids takes place at considerable frequencies resulting in the formation of mosaic plasmid structures (Boyd *et al.*, 1996). A prerequisite for these recombination events to occur is that surface exclusion is at least partially repressed. Evidence for recombination between F-like plasmids indicates that surface exclusion is not an insurmountable barrier to the entry of an F-like plasmid into a cell already harbouring a plasmid of the same incompatibility group. Until now recombination between plasmids belonging to the IncP-1 group has not been demonstrated.

Waste-water treatment plants receive water with bacteria that were previously exposed to antibiotics and/or xenobiotics from households, hospitals, animal husbandries, agriculture or industry, and are considered to be hot-spots for horizontal gene transfer because of their nutritional richness and high bacterial densities. Thus, it is not surprising that several studies have described the isolation of mobile plasmids from sewage water and the activated sludge compartment of waste-water treatment plants (Top *et al.*, 1994; Blázquez *et al.*, 1996; Heuer *et al.*, 2002; van Overbeek *et al.*, 2002; Smalla & Sobczyk, 2002). Dröge *et al.* (2000) isolated 12 distinct plasmids, designated pB1–pB12, from activated sludge bacteria by using the exogenous plasmid isolation method. These plasmids conferred various antibiotic-resistance patterns on their hosts and 10 of the 12 plasmids were categorized as members of incompatibility group IncP-1. One of these plasmids, namely pB4, was first chosen for complete sequencing because partial sequencing of this plasmid revealed that it encodes a putative new multidrug efflux system similar to efflux systems of the pathogenic bacterium *Pseudomonas aeruginosa* (Dröge *et al.*, 2000). The complete pB4 sequence shows that it consists of an IncP-1 β backbone that is not very similar to R751 nor to the other sequenced degradative plasmids pTSA and pADP-1. Plasmid pB4 is loaded with a chromate-resistance transposon, the streptomycin-resistance transposon Tn5393c, the β -lactamase gene *bla*_{NPS1} flanked by relics of integron-specific sequences and a gene region for a tripartite antibiotic efflux system of the RND- (resistance-nodulation-division), MFP- (membrane fusion protein), OMF (outer membrane factor) type (Tauch *et al.*, 2003).

Plasmid pB10, isolated from the same waste-water treatment plant as pB4, confers resistance to the antimicrobial agents amoxicillin, streptomycin, sulfonamides and tetracycline and to inorganic mercury ions. Partial sequencing of the pB10 replication initiation gene *trfA1* revealed that this sequence is very closely related to the corresponding sequences of the degradative plasmids pTSA and pADP-1.

Here we report the detailed sequence analysis of the IncP-1 β plasmid pB10 and compare its genome with other sequenced IncP-1 β plasmid genomes. Plasmid pB10 was shown to contain five distinct mobile genetic elements, most of which carry resistance genes. It has a mosaic backbone structure, indicative of a recombination event between different IncP-1 β plasmids. In addition, several features of pB10 shed new light on its evolutionary relationship to degradative and other resistance plasmids of the IncP-1 β group.

METHODS

Bacterial strains and growth conditions. *Escherichia coli* DH5 α *mcr* (Grant *et al.*, 1990) containing the multiresistance plasmid pB10 was grown at 37 °C in Luria Broth (LB) medium supplemented as needed with either 40 μ g streptomycin ml⁻¹ or 5 μ g tetracycline ml⁻¹. For solid media, 15 g agar per litre of medium were added. Indicator medium for strains expressing an active β -galactosidase was supplemented with 40 μ g X-Gal ml⁻¹ (final concentration).

Standard DNA techniques. Plasmid DNA from the pB10-containing *Escherichia coli* DH5 α *mcr* derivative was isolated with the Nucleobond Kit PC100 on AX 100 columns (Macherey-Nagel) according to the protocol supplied by the manufacturer. Recombinant pGEM-T-Easy derivatives were isolated using the QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer's instructions. DNA was extracted from agarose gels with the Sephaglass BandPrep Kit (Amersham Pharmacia Biotech) and purified on Sephacryl MicroSpin S-400 HR columns (Amersham Pharmacia Biotech). Restriction enzyme digestion, agarose gel electrophoresis, DNA cloning and transformation of *Escherichia coli* DH5 α were carried out according to Sambrook *et al.* (1989).

Subcloning of DNA fragments generated by PCR. A DNA fragment covering the pB10 *ssb-trbA* intergenic region was amplified by using the primers *trbA*-1 (5'-GCAATGTCCTCCATCACCTT-3') and *ssb*-1 (5'-GGTGCCAGGTATTCGATTT-3') binding in the *trbA* and *ssb* coding regions, respectively, and cloned into the vector pGEM-T-Easy (T-cloning vector; Promega) according to the pGEM-T-Easy Vector Systems protocol supplied by the manufacturer (Promega). Recombinant pGEM-T-Easy derivatives were characterized by restriction analysis and by sequencing with standard sequencing primers.

Construction of a shotgun library and DNA sequencing of pB10. Purified pB10 plasmid DNA was randomly fragmented by hydro-shearing and the 1.3 to 2.0 kb size fractions were cloned into the sequencing vector pGEM-T-Easy (MWG Biotech AG). Plasmid DNA was prepared from the *Escherichia coli* shotgun clones by an automated alkaline lysis with the BioRobot 9600 (Qiagen).

Sequencing reactions using the dye-terminator and dye-primer chemistries were separated on Prism ABI 377 (Applied Biosystems) and Li-Cor IR 4200 (Li-Cor) DNA sequencers, respectively.

Sequencing reads were assembled using the STADEN (GAP4) software package (Staden, 1996). Gap closure and polishing of the sequence were achieved by primer walking with custom-made primers. This approach resulted in a single, circular molecule with a total length of 64 508 bp.

DNA sequence analysis and annotation. Annotation of the finished pB10 sequence was done by using the GENDB (version 2.0) Annotation Tool (Meyer *et al.*, 2003) as described by Tauch *et al.* (2003). Repeat regions within the pB10 sequence were identified and analysed by using REPUTER software (Kurtz *et al.*, 2001). Global amino acid sequence similarities were determined by using the ALIGN PLUS 4 (version 4.10) software package incorporated in the CLONE MANAGER PROFESSIONAL SUITE (Scientific & Educational Software) with the scoring matrix 'Standard Linear'. Multiple sequence alignments and phylogenetic analyses were done by using the CLUSTAL W (version 1.6) software and the PHYLIP package (3.5) incorporated in the OPEN GENOME ENVIRONMENT (OGenE, IMB, Jena, Germany). The annotated sequence of pB10 has been deposited in the EMBL database under accession number AJ564903.

RESULTS AND DISCUSSION

Resistance plasmid pB10 possesses a characteristic IncP-1 β -specific backbone loaded with five distinct mobile genetic elements

To further characterize the IncP-1 β resistance plasmid pB10, isolated from a bacterial community residing in the activated sludge compartment of a waste-water treatment plant, the complete nucleotide sequence of the plasmid was established. This was achieved by applying a shotgun sequencing approach, which resulted in a circularly closed sequence of 64 508 bp (Fig. 1) with a mean G + C content of 64.2 mol%. It thus appears that the size of pB10 is around 11 % larger than estimated by restriction analysis (Dröge *et al.*, 2000). Annotation of the sequence data revealed that pB10 contains 65 complete coding regions and seven truncated transposase genes (Δ *tnpA*) (Tables 1 and 2). An inventory of the coding sequences (cds) showed that 29 (40.3 %) are involved in mating-pair formation (*trb* genes) and conjugative transfer (*tra* genes), 10 (13.9 %) are involved in regulatory processes, seven (9.7 %) are involved in plasmid maintenance, stable inheritance and replication, seven (9.7 %) are involved in antibiotic and mercury resistance, three (4.2 %) are involved in site-specific recombination and two (2.8 %) are involved in transport processes. No function could be assigned to seven (9.7 %) coding sequences. It appeared that 63.3 % of the pB10 nucleotide sequence is occupied by IncP-1 β plasmid-specific backbone genes. These backbone regions are separated by the accessory genes, which form the 'genetic load' of the plasmid, and were inserted at positions very similar or identical to those of other IncP-1 β plasmids. The 'genetic load' of pB10 (about 37 % of the total sequence length) is composed of an intact class I integron, which is inserted between the *trb* (mating-pair formation) and *tra* (conjugative transfer) regions, and four class II (Tn3 family) transposable elements, which were found downstream of the replication gene *trfA1*. The characteristics of

Table 1. Coding sequences of the pB10 backbone segments and comparison of the corresponding gene products with those of the IncP-1 β plasmids R751, pADP-1, pB4 and pTSA

Sequences of the IncP-1 β plasmids R751, pADP-1 and pB4 are available under GenBank accession numbers U67194, U66917 and AJ431260, respectively. The accession number for the partial sequence of the IncP-1 β plasmid pTSA is AF311437.

Coding sequence	pB10 coordinates (5'→3')	G+C content (mol%)	Protein			Identity, similarity (%) of pB10 at the gene-product level to:				Best GenBank Protein hit ref. no.
			No. of residues	Molecular mass (kDa)	pI value	R751	pADP-1	pB4	pTSA	
<i>trbA</i>	97–459	58·68	120	13·32	8·87	99, 100	100, 100	90, 95	84, 85	AAK50320
<i>trbB</i>	769–1731	61·99	320	35·04	6·58	99, 99	100, 100	93, 96	100, 100	AAK50319 AAK38002
<i>trbC</i>	1748–2212	66·88	154	16·35	7·52	98, 98	100, 100	90, 94	100, 100	AAK50318 AAK38003
<i>trbD</i>	2216–2527	63·00	103	11·81	11·60	98, 98	100, 100	97, 99	100, 100	AAK50317 AAK38004
<i>trbE</i>	2524–5082	63·81	852	93·98	5·97	99, 99	100, 100	92, 96	100, 100	AAK50316 AAK38005
<i>trbF</i>	5079–5861	65·64	260	28·73	9·83	99, 99	100, 100	82, 89	99, 99	AAK50315
<i>trbG</i>	5858–6778	63·95	306	33·42	8·99	99, 99	100, 100	83, 90	100, 100	AAK50314 AAK38007
<i>trbH</i>	6781–7269	70·55	162	17·00	9·67	99, 99	100, 100	71, 80	100, 100	AAK50313 AAK38008
<i>trbI</i>	7274–8695	67·37	473	49·38	9·35	99, 99	100, 100	75, 83	100, 100	AAK50312 AAK38009
<i>trbJ</i>	8716–9480	60·92	254	27·44	9·45	100, 100	100, 100	75, 84	100, 100	AAC64451 AAK50311 AAK38010
<i>trbK</i>	9490–9717	62·28	75	7·99	9·49	98, 98	98, 98	61, 70	100, 100	AAK38011
<i>trbL</i>	9728–11446	68·18	572	55·62	4·70	99, 99	100, 100	78, 83	99, 99	AAK50309
<i>trbM</i>	11464–12051	67·52	195	21·54	9·27	90, 93	100, 100	79, 85	100, 100	AAK50308 AAK38013
<i>trbN</i>	12065–12700	69·18	211	22·75	9·86	99, 99	100, 100	85, 87	100, 100	AAK50307 AAK38014
<i>trbO</i>	12729–12995	63·37	88	9·90	10·60	100, 100	–	87, 92	100, 100	AAC64456 AAK38015
<i>trbP</i>	12995–13693	68·24	232	25·67	10·48	99, 99	–	81, 90	100, 100	AAK38016
<i>upf30.5</i>	13709–14140	65·97	143	15·09	8·89	99, 99	–	69, 78	100, 100	AAK38017
<i>traC</i>	23675–19329	68·62	1448	158·98	5·35	99, 99	99, 99	50, 55	–	AAC64468 AAK50260
<i>traD</i>	24068–23679	72·56	129	13·60	3·54	100, 100	100, 100	53, 68	–	AAC64471 AAK50259
<i>traE</i>	26153–24090	67·10	687	74·87	9·29	85, 86	100, 100	92, 96	–	AAK50258
<i>traF</i>	26701–26165	66·29	178	18·85	9·91	100, 100	100, 100	84, 92	–	AAC64473 AAK50257
<i>traG</i>	28611–26698	66·09	637	69·89	8·85	99, 100	100, 100	92, 95	–	AAK50256
<i>traH</i>	29302–28910	67·68	130	13·94	4·01	100, 100	100, 100	76, 81	–	AAC64476 AAK50255
<i>traI</i>	30848–28608	66·89	746	82·04	10·62	99, 99	99, 99	84, 90	–	AAC64475 AAK50254
<i>traJ</i>	31257–30883	66·93	124	14·21	8·89	85, 86	100, 100	86, 90	–	AAK50253
<i>traK</i>	31631–32029	63·16	132	14·62	9·89	100, 100	100, 100	31, 46	–	AAC64479 AAK50252
<i>traL</i>	32029–32754	62·81	241	26·38	5·44	99, 100	99, 100	92, 96	–	AAC64480 AAK50251
<i>traM</i>	32754–33194	67·57	146	15·58	5·26	100, 100	100, 100	81, 90	–	AAC64481

Table 1. cont.

Coding sequence	pB10 coordinates (5'→3')	G+C content (mol%)	Protein			Identity, similarity (%) of pB10 at the gene-product level to:				Best GenBank Protein hit ref. no.
			No. of residues	Molecular mass (kDa)	pI value	R751	pADP-1	pB4	pTSA	
<i>traM</i>										AAK50250
<i>traN</i>	34 050–33 397	69·27	217	24·03	8·88	97, 97	100, 100	34, 40	–	AAK50249
<i>traO</i>	34 426–34 079	58·33	115	12·49	10·19	99, 99	100, 100	78, 86	–	AAK50248
<i>kfrA</i>	35 628–34 597	74·71	343	36·78	5·02	99, 99	99, 100	60, 71	–	AAK50247
<i>korB</i>	36 856–35 807	67·33	349	38·14	4·70	99, 100	99, 99	86, 89	–	AAC64419
<i>incC1</i>	37 935–36 853	64·73	360	38·05	9·78	88, 91	88, 91	94, 96	–	CAD24327
<i>incC2</i>	37 617–36 853	65·23	254	27·58	8·67	92, 94	–	94, 98	–	CAD24328
<i>korA</i>	37 922–37 614	63·43	102	11·11	10·30	91, 94	91, 94	94, 97	–	CAD24329
<i>kleF</i>	38 358–38 029	63·33	109	11·95	5·02	49, 52	50, 52	90, 93	–	CAD24330
<i>kleE</i>	38 686–38 360	63·30	108	11·86	10·30	80, 86	68, 73	97, 98	–	CAD24331
<i>kleA</i>	39 131–38 895	67·09	78	8·60	4·69	71, 73	71, 73	94, 97	–	CAD24332
<i>korC</i>	39 546–39 289	68·22	85	9·32	6·85	87, 92	87, 92	78, 89	–	AAC64428 AAK50238
<i>klcB</i>	40 771–39 563	69·56	402	43·18	6·00	81, 86	25, 28	94, 96	–	CAD24334
<i>klcA</i>	41 474–41 046	67·60	142	15·62	4·34	92, 95	92, 95	98, 98	–	CAD24335
<i>din</i>	41 647–41 910	64·77	87	9·26	5·60	–	–	86, 86	–	CAD24336
<i>xf2080</i>	41 897–42 178	68·44	93	10·74	7·66	–	–	–	–	G82603
<i>trfA1</i>	64 103–62 883	65·77	406	45·77	9·08	97, 97	74, 74	80, 86	99, 99	AAK37999
<i>ssb</i>	64 491–64 150	59·65	113	12·65	7·41	100, 100	100, 100	88, 90	100, 100	AAC64441 AAK50321 AAK38000

(Hedges *et al.*, 1974). A reasonably informative restriction map is available for R906 (Smith & Thomas, 1987); therefore, the positions of restriction sites for *Bam*HI, *Bgl*II, *Eco*RI, *Hind*III, *Pst*I, *Sal*I, *Sma*I and *Sst*II were determined for pB10 based on its DNA sequence. The pattern obtained is identical to that of R906 except in the region that encodes tetracycline resistance. The remaining clusters of restriction sites associated with the two blocks of phenotypic determinants suggest that the only difference between pB10 and R906 is the presence of a tetracycline-resistance determinant. The identity of at least part of the backbones of pB10 and R906 is confirmed at the DNA sequence level in the *trfA*–*oriV* region (Smith *et al.*, 1993), which is the only R906 sequence available to date. However, due to the limited number of restriction sites in the IncP-1 β backbone regions, it is impossible to know whether this identity extends right across the backbone. That is, pB10 and R906 may have a common sector, but may have undergone different recombination events of the type described below.

The pB10 backbone shows a mosaic structure with important parts being closely related to IncP-1 β degradative plasmids

It appears that the pB10 backbone segments for conjugative transfer and replication on the one hand and stable inheritance on the other hand are derived from different ancestral plasmids. This may be explained by a recombination event between two different IncP-1 β plasmids residing

in the same cell. The pB10 regions containing the *trb* (*trbA*–*trbN*) and *tra* genes (*traC*–*traO*) are very closely related to the corresponding parts of the degradative IncP-1 β plasmids pADP-1 (responsible for atrazine catabolism; Martinez *et al.*, 2001) and pTSA (encoding genes for *p*-toluenesulfonate degradation; Tralau *et al.*, 2001), as well as to R751 (Thorsted *et al.*, 1998), whereas the sequence similarity with the *trb* and *tra* segments of the IncP-1 β resistance plasmid pB4 from an uncultured activated sludge bacterium (Tauch *et al.*, 2003) was much lower (Table 1 and Fig. 3). Similar results were obtained when the replication modules *trfA1*–*ssb* of the different IncP-1 β plasmids were compared (Table 1 and Fig. 3). A completely different picture emerges, however, when analysing the stable inheritance operons *klcAB*–*korC* and *kle*. In this region, the DNA sequences of pB10 and pB4 are most similar, while the corresponding DNA sequences of pADP-1 and R751 are much less similar to the pB10 sequence (Table 1, Figs 2 and 3). This is mainly due to the absence of *kleB* and *kleG* and an abbreviated *kleF* gene in pB10 and pB4 as compared to pADP-1 and R751. In addition, the pB10 and pB4 *klcA*–*klcB* intergenic region is longer than the corresponding region of pADP-1 and R751 (Fig. 2). The evolutionary relationship of the IncP-1 β plasmids described above is also apparent at the gene-product level. This mosaic structure of the pB10 backbone, which could be explained by a recombination event between two different IncP-1 β plasmids, would require the presence of two

Table 2. Coding sequences of the pB10 'genetic load' regions and comparison of the corresponding gene products with the closest relatives

Coding sequence	pB10 coordinates (5'→3')	G+C content (mol%)	Protein			Identity, similarity (%) of pB10 at the gene-product level	Best GenBank Protein hit ref. no.
			No. of residues	Molecular mass (kDa)	pI value		
<i>orf5</i>	14 904–14 404	65.07	166	18.31	5.17	100, 100; Tn2000	AAG45724
<i>sul1</i>	15 871–15 032	61.55	279	30.13	5.69	100, 100; R100	NP_052895
<i>qacEΔ1</i>	16 212–15 865	50.00	115	12.33	10.48	100, 100; R100	NP_052896
<i>orfE</i> -like	16 559–16 318	63.64	79	7.93	10.58	70, 74; Tn1696 of R1033	AAB60001
<i>oxa-2</i>	17 434–16 607	50.00	275	31.69	9.63	100, 100; R46	NP_511223
<i>int11</i>	17 592–18 605	61.24	337	38.38	10.63	100, 100; In2 of R100	NP_052898
' <i>tnpA</i> _{Tn501-like} *	43 762–43 523	64.17	79	–†	–	100, 100; <i>R. metallidurans</i> hypothetical protein	ZP_00023731
' <i>tnpA</i> _{IS1071} *	46 065–43 906	59.95	719	–	–	100, 100; IS1071	Q04222
' <i>tnpA</i> _{Tn1721} *	46 066–46 932	65.40	288	–	–	100, 100; Tn1721	CAD11600
<i>tetR</i>	47 914–47 264	63.90	216	23.32	5.06	100, 100; Tn1721	JQ1478
<i>tetA</i>	48 020–49 219	63.67	399	42.21	9.93	100, 100; Tn1721	JQ1479
<i>pecM</i> -like	50 135–49 251	58.98	294	31.15	11.76	61, 75; <i>A. tumefaciens</i> C58 regulator protein PecM	AAL41295
' <i>tnpA</i> _{Tn1721} *	50 669–52 420	65.58	583	–	–	99, 100; Tn1721	CAD11600
<i>tnpA'</i> _{IS1071} ‡	52 800–52 453	57.18	116	–	–	100, 100; IS1071	Q04222
<i>tnpA'</i> _{Tn5393c} ‡	54 758–52 947	62.58	604	–	–	100, 100; pB4	CAD24402
<i>tnpR</i> _{Tn5393c}	54 953–55 498	60.44	181	22.27	10.38	100, 100; pB4	CAD24403
<i>strA</i>	55 564–56 367	56.22	267	29.57	4.60	100, 100; pB4	CAD24404
<i>strB</i>	56 367–57 203	55.91	278	30.82	4.61	100, 100; pB4	CAD24405
<i>tnpA'</i> _{Tn501-like} ‡	57 573–57 310	63.26	–	–	–	100, 100; <i>Pseudomonas</i> sp. transposase	CAC14696
<i>tnpR</i> _{Tn501-like}	58 136–57 576	62.03	186	21.58	10.30	87, 89; Tn501	CAA77327
<i>orf-2</i>	59 256–58 267	62.22	329	35.69	4.31	99, 99; Tn501	CAA77326
<i>merE</i>	59 489–59 253	64.56	78	8.41	9.06	100, 100; Tn501	CAA77325
<i>merD</i>	59 851–59 486	69.67	121	13.02	4.81	99, 99; Tn501	CAA77324
<i>merA</i>	61 554–59 869	65.66	561	58.70	5.50	99, 99; Tn501	CAA77323
<i>merP</i>	61 901–61 626	61.59	91	9.49	10.10	98, 98; Tn501	CAA77322
<i>merT</i>	62 318–61 914	59.01	134	12.50	10.10	86, 86; Tn501	CAA77321
<i>merR</i>	62 336–62 770	60.92	144	15.76	6.37	100, 100; Tn501	CAA77320

*3' part of a truncated *tnpA* transposase gene.

†–, Not determined due to truncation of the gene.

‡5' part of a truncated *tnpA* transposase gene.

distinct IncP-1 β plasmids in the same cell. This seems to be possible for IncP-1 plasmids if both plasmids are temporarily selected for, since they could both enter the cell due to weak surface exclusion (Lessl *et al.*, 1991; Thorsted *et al.*, 1998). The recombination event most probably occurred in a region which is approximately 232 bp downstream of the *incC2* start codon since the 3' part of *incC2* is very similar to '*incC2*' of pADP-1 whereas the 5' part of the gene is more related to '*incC2*' present on pB4. The hypothesis that the pB10 and pB4 stable inheritance operons *klcAB*–*korC* have a common ancestor is also supported by the presence of a gene encoding a DNA-damage-inducible-like (Din) protein homologous to *Xylella fastidiosa* XF2081 upstream of *klcA* in both plasmids. Such XF2081-like genes were not found on other IncP-1 β plasmids. In this context, it is interesting

to note that downstream of the XF2081 homologous gene an open reading frame (ORF) similar to the *X. fastidiosa* gene XF2080, encoding a conserved hypothetical protein, was found in the pB10 sequence, whereas this ORF was replaced in pB4 by insertion of a multidrug efflux gene cluster (*nfxB*–*mexCD*–*oprJ*). Unfortunately, the DNA sequence of the stable-inheritance and central control region is not known so far for the degradative IncP-1 β plasmid pTSA, so that this region cannot be compared with the pB10 sequence. Recombination between plasmid backbones of closely related plasmids has been shown for F-like plasmids from the *Escherichia coli* reference collection (ECOR) (Boyd *et al.*, 1996) but, to our knowledge, we present here the first evidence for recombination of backbone regions as a mechanism of evolution of IncP-1 β plasmids.

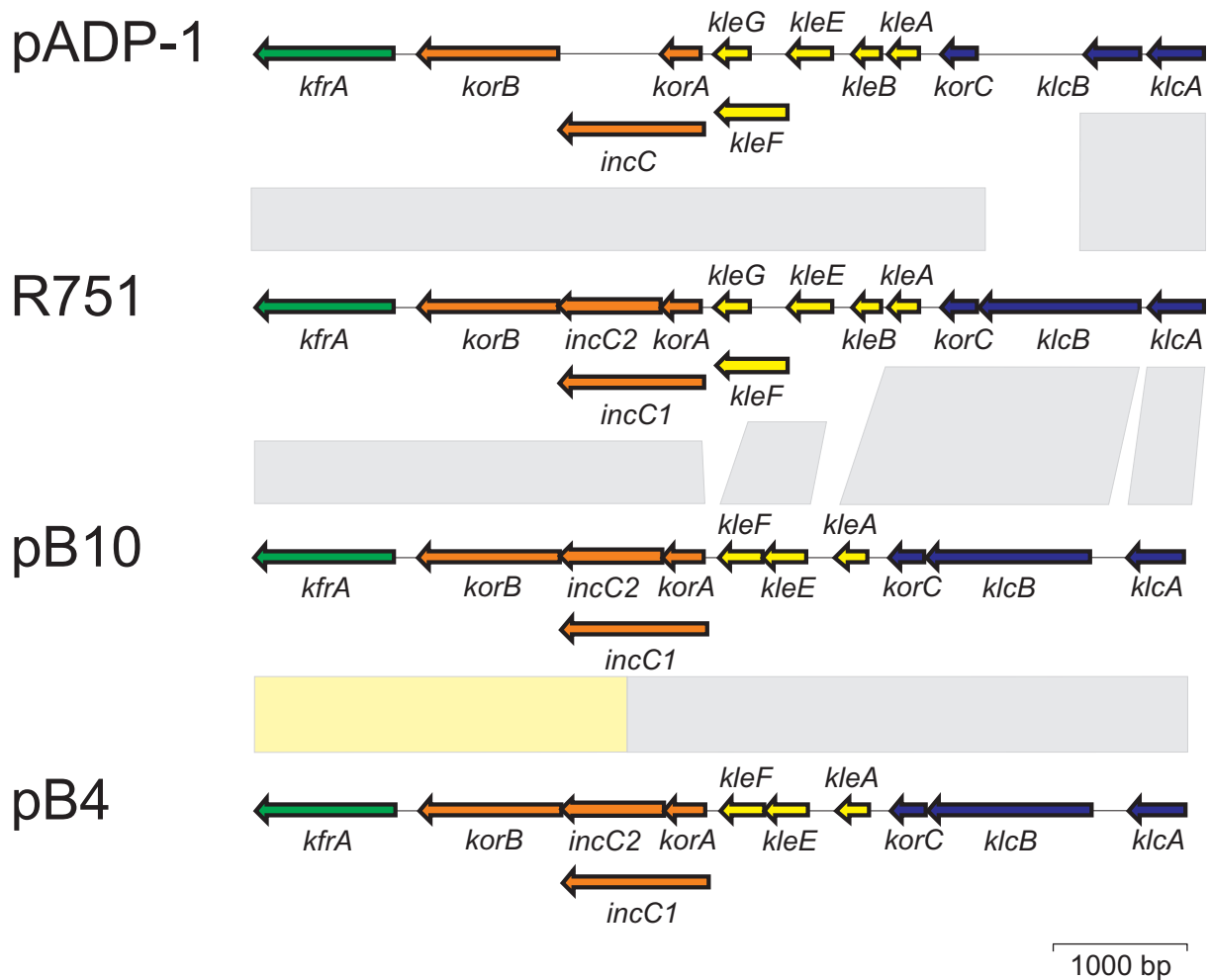


Fig. 2. Comparison of the genetic organization of the central control/stable-inheritance regions located on the IncP-1 β resistance plasmids pADP-1, R751, pB10 and pB4. Coding regions are marked by arrows indicating the direction of transcription. The different putative transcriptional units of the central control/stable-inheritance regions of the IncP-1 β plasmids pADP-1, R751, pB10 and pB4 are presented in different colours: *klcAB-korC*, dark blue; *kle*, yellow; *korA-incC-korB*, orange; *kfrA*, green. Homologous regions are indicated by grey areas. The pB10 stable inheritance operons *klcAB-korC* and *kleAEF* are most similar to those of the resistance plasmid pB4. The light-yellow area below the pB10 map indicates a segment which is less well conserved as compared to the corresponding pB4 region but shows the highest degree of similarity to *incC-korB-kfrA* of the degradative plasmid pADP-1. This observation can be explained by a recombination event between different IncP-1 β plasmids. The organization of the *korC-kle* region of the degradative plasmid pADP-1 is very similar to the type shown for the resistance plasmid R751. Accession numbers for pADP-1, R751 and pB4 are U66917, U67194 and AJ431260, respectively.

Resistance plasmid pB10 contains a complete class 1 integron

An intact class 1 integron, potentially capable of integrating and disseminating resistance gene cassettes, was identified between the conjugative transfer gene *traC* and *upf30.5*, which is located downstream of the mating-pair-formation gene *trbP* (Fig. 1). The combination of the gene cassettes present in the pB10 integron has not been described before. A 25 bp inverted repeat (IR) which is identical to the terminal IR of In2 located on plasmid R100 (GenBank

accession no. AP000342) was found 178 bps downstream of the *intI1* gene, encoding a site-specific integrase, and seems to be the boundary of the integron since typical IncP-1 β backbone sequences were found beyond that point. The 3' region of the pB10 integron is composed of the genes *qacE Δ 1*, encoding a small exporter protein, *sul1*, encoding a dihydropteroate synthetase, conferring sulfonamide resistance, and *orf5*, encoding a putative acetyltransferase. These genes are typically present in the 3'-conserved segments of class 1 integrons (Stokes & Hall, 1989). Sequence identity

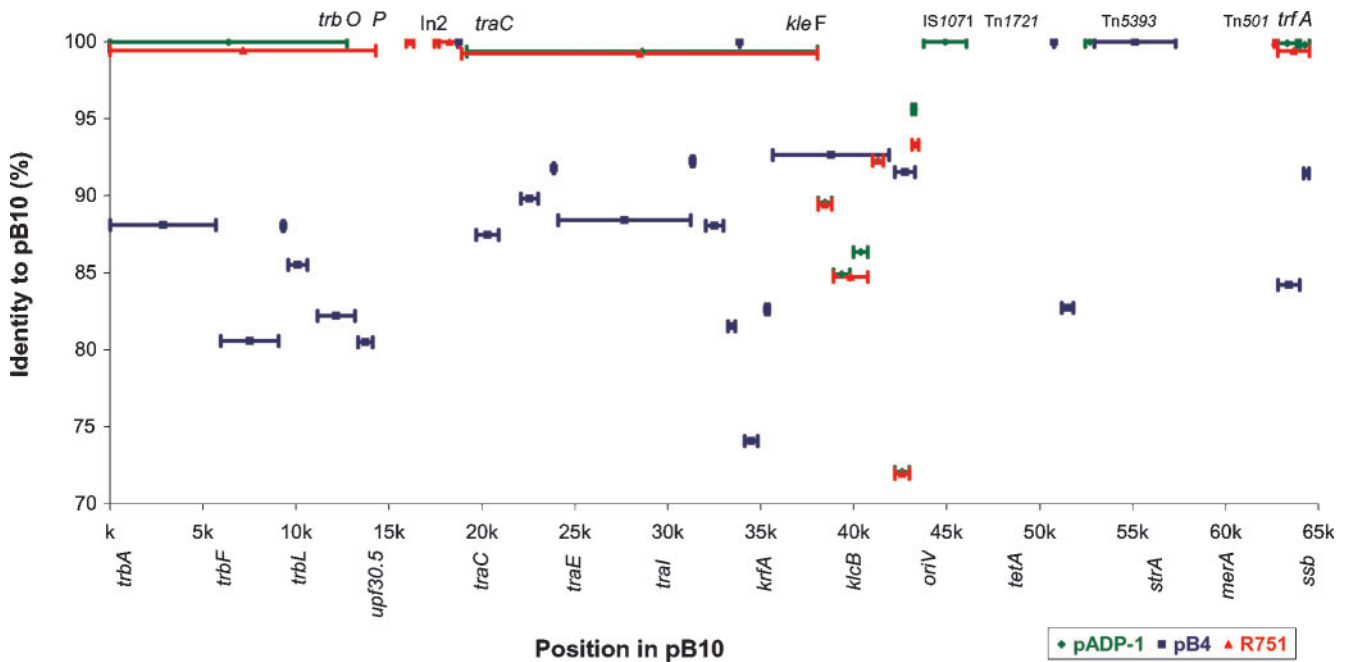


Fig. 3. Whole plasmid sequence comparison of pB10 with R751, pADP-1 and pB4. The program MUMMER 2.12 (<http://www.tigr.org/software/mummer/>) (Delcher *et al.*, 2002) was used to find clusters of matching sequences with 15 bp minimum length of matches, 800 bp maximal separation between matches in a cluster, and 15% error-rate cut-off in combining matches to a cluster.

to In2 extends for 101 bp downstream of *orf5* but a characteristic IR sequence motif could not be found at this end of the integron. The *oxa2* cassette encodes a β -lactamase of the oxacillin-hydrolysing type, conferring resistance to amoxicillin, and is identical to both copies of *oxa2* present in the integron In1 located on the IncN resistance plasmid R46 (Stokes & Hall, 1992), which contains the gene cassette arrangement *oxa2*–*aadA1*–*oxa2*–*orfD*. The *oxa2* cassette was also found on the resistance plasmid pBP11 from *Salmonella typhimurium* (Nücken *et al.*, 1989) and on the multidrug-resistant conjugative plasmid pBWH301 from *Enterobacter aerogenes* and *Enterobacter cloacae* (Bunny *et al.*, 1995). The integron of pB10 may have acquired the *oxa2* gene cassette quite recently, since the *oxa2* G+C content of 50 mol% differs considerably from the mean G+C content of the whole plasmid (64.2 mol%). The *orfE*-like gene cassette downstream of *oxa2* on pB10 encodes a putative protein that is 70% identical to the *orfE* gene product of *P. aeruginosa*, the function of which is unknown (GenBank accession no. U12338).

An integron-like element and relics of an integron were also found in the *trb*–*tra* intergenic regions of the IncP-1 β plasmids R751 and pB4. The prototype IncP-1 β plasmid R751 harbours an integron termed Tn5090 (Radström *et al.*, 1994) that possesses two tandemly arranged mobile gene cassettes, not found on pB10. The segment downstream of the gene cassettes contains the *qacE* gene but a *sul1* gene is missing in this Tn5090 element. It thus appears that pB10

is the only IncP-1 β plasmid analysed at the sequence level so far that contains a typical class 1 integron with completely conserved 5' and 3' segments. As deduced from its restriction pattern, R906 most probably carries exactly the same element (see above). Tn5090 on R751 carries a complete transposition module composed of the genes *tniA*, *tniB* and *tniC* downstream of *qacE*. This module could not be found in the corresponding region of the pB10 integron. These observations support the view that ancestors of plasmids pB10 and R751 acquired their integron elements by independent events. This assumption is also supported by the fact that the integrons of pB10 and R751 were inserted in different orientations with respect to the transcriptional direction of the *traC* gene. In contrast to these two IncP-1 β plasmids, plasmid pB4 contains only remnants of an integron structure flanking the class D oxacillinase gene *bla*_{NPS1}, which is located downstream of the conjugative transfer *tra* operon (Tauch *et al.*, 2003). Obviously, pB4 contains neither a functional integron nor a Tn5090-like element between the *tra* and *trb* regions. The degradative IncP-1 β plasmids pTSA (Tralau *et al.*, 2001) and pADP-1 (Martinez *et al.*, 2001) do not have any integron structures, but their degradative genes are integrated in the same region as the class 1 integron in pB10 and the Tn5090 element in R751, i.e. between the *trb* and *tra* regions.

In summary, pB10 contains a typical and complete class 1 integron with a unique combination of gene cassettes.

pB10 contains a chimeric mercury-resistance transposon with the *mer* genes and the transposition module derived from different sources

Plasmid pB10 contains a mercury-resistance (*mer*) transposon (shown in Figs 4 and 5) that seems to be a chimera, with the transposition module and the *mer* genes, respectively, derived from a transposon residing in the genome of *Ralstonia metallidurans* and another Tn501-like transposon. The pB10 *mer* transposon is inserted between *oriV* and the replication initiation gene *trfA1*. The transposase gene *tnpA_{mer}* of this transposon is truncated and disrupted by the insertion of a streptomycin-resistance transposon (see below). The N- and C-terminal portions of the *tnpA_{mer}* gene product (88 and 79 aa, respectively) are identical to the homologous regions of a hypothetical protein (GenBank accession no. ZP_00023731) encoded in the genome of the metal-resistant bacterium *R. metallidurans*. This hypothetical protein on its part is similar to the *tnpA* gene product of Tn501 located on plasmid pVS1 of *P. aeruginosa* (GenBank accession no. Z00027) and therefore most probably is also a transposase. The central part of the pB10 *tnpA_{mer}* gene was presumably deleted during transposition of another transposable element into this gene. Upstream of the pB10 *tnpA_{mer}* gene, a *tnpR* gene encoding a resolvase was found. In summary, the transposition module

tnpA_{mer}-tnpR of the pB10 mercury-resistance transposon shows the highest identity (99.7% at the DNA sequence level) to a corresponding module present in the genome of *R. metallidurans* (GenBank accession no. NZ_AAAI01000299). Surprisingly, this *R. metallidurans* transposition module is not associated with mercury-resistance (*mer*) genes. The stretch of DNA upstream of the pB10 *tnpA_{mer}-tnpR* transposition module is nearly identical to the corresponding region of Tn501 present on the *P. aeruginosa* plasmid pVS1 (GenBank accession no. Z00027) and on the *Shigella flexneri* virulence plasmid pWR501 (Venkatesan *et al.*, 2001) (99.8% identity over a sequence length of 4504 bp). This region contains the mercury-resistance genes *merR*, *merT*, *merP*, *merA*, *merD*, *merE* and *orf-2* which encode a repressor (MerR), an integral membrane protein for mercuric transport (MerT), a periplasmic mercury ion-binding protein (MerP), a mercuric reductase (MerA), a co-regulator protein (MerD) and a protein of unknown function (MerE) (see Table 2). The deduced *orf-2* gene product possesses an EAL domain (Pfam accession no. PF00563), which was named after its conserved residues. This domain was found in diverse bacterial signalling proteins (Galperin *et al.*, 2001) and might form the active site of a diguanylate phosphodiesterase. It might be speculated that Orf-2 plays a role in a signalling cascade to MerR or MerD. In conclusion, the transposition module and the *mer* gene region of the pB10 *mer* transposon

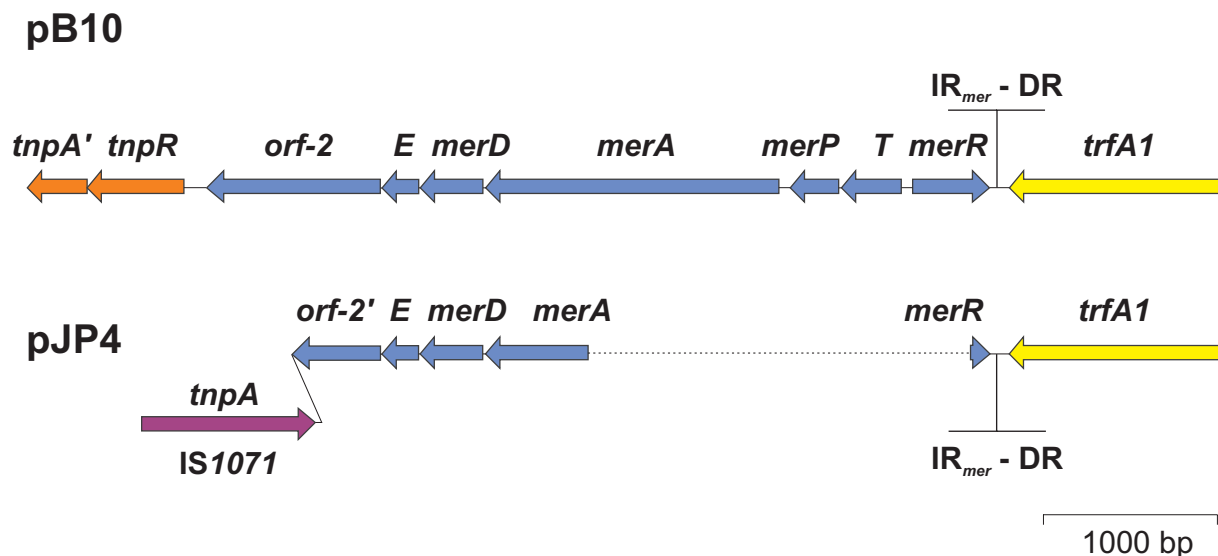


Fig. 4. Comparison of Tn501-like mercury-resistance (*mer*) transposon insertions in the IncP-1 β resistance plasmid pB10 and the IncP-1 β degradative plasmid pJP4. Genes are marked by arrows indicating the direction of transcription. Both plasmids contain very similar insertions of Tn501-like *mer* transposons in the same target site. The DNA sequence of the *merR-trfA1* intergenic region containing the IR (*IR_{mer}*) sequence and the target site duplication (direct repeat, DR) of the transposon is identical in both plasmids. The DNA sequence of the pJP4 region containing '*merA-merD-merE-orf2'*' is also identical as compared to the corresponding region of pB10. The dashed line in the pJP4 map indicates a region that has not been sequenced. In pJP4, a copy of the insertion sequence element IS1071 is inserted in *orf-2*. In pB10, the transposition module (*tnpR-tnpA'*) of the *mer* transposon is located downstream of *orf-2*. DNA sequence data for pJP4 were taken from the GenBank database entries PJPTRN501 and AF225973.

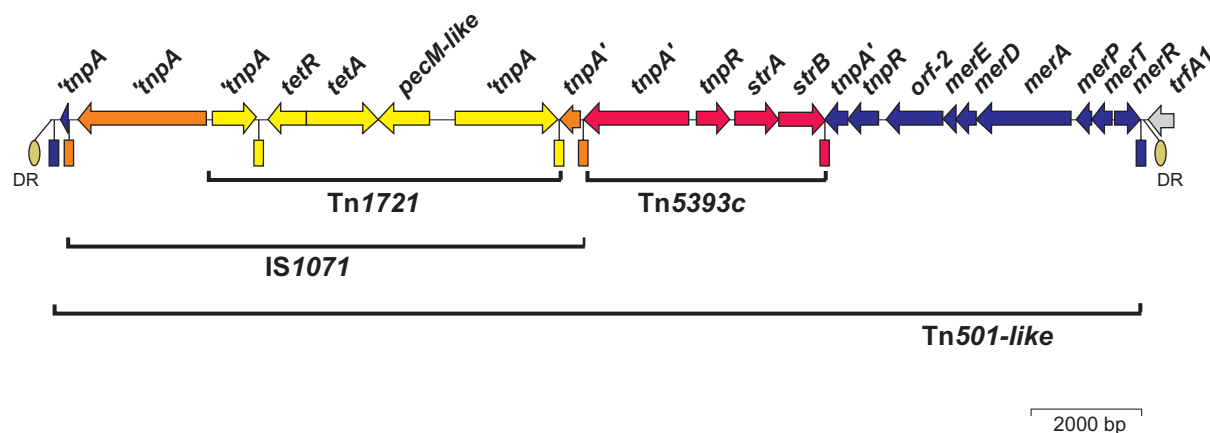


Fig. 5. The pB10 'genetic load' region downstream of the replication initiation gene *trfA1* is composed of four truncated derivatives of class II transposable elements. The different transposable elements and the corresponding IR sequences (marked by rectangles) are distinguished by different colours: Tn501-like mercury-resistance (*mer*) transposon, blue; Tn5393c streptomycin-resistance transposon, red; insertion sequence element IS1071, orange; Tn1721 tetracycline-resistance transposon, yellow. The target site duplication (direct repeat, DR) of the Tn501-like *mer* transposon is marked. Further details of the genes are given in Table 2.

most probably were derived from different sources and combined by homologous or site-specific recombination.

Insertions of Tn501-like transposons downstream of the replication gene *trfA1* seem to be a common feature of IncP-1 β plasmids, although the prototype IncP-1 β plasmid R751 only contains a short relict of a Tn501-like element with no functional mercury-resistance genes. When compared to pB10, plasmid R751 acquired the Tn501-like insertion by an independent event since the orientation of the transposon is the inverse of that in pB10 (Thorsted *et al.*, 1998). The second IncP-1 β resistance plasmid pB4 contains only remnants of a class II transposon (Tn501-like) inserted downstream of the *trfA1* gene and no *mer* genes were found on this plasmid (Tauch *et al.*, 2003). In contrast, the IncP-1 β degradative plasmid pTSA harbours a complete mercury-resistance operon inserted between *oriV* and the *trfA1* gene, but the detailed structure of this pTSA *mer* operon has not been analysed until now (Tralau *et al.*, 2001). Likewise, a functional mercury-resistance region is part of a complex region inserted between the *trb* and *tra* modules in the atrazine catabolic plasmid pADP-1 from *Pseudomonas* sp. strain ADP (Martinez *et al.*, 2001). The pADP-1 *mer* region contains the genes *merE*, *merD*, *merA*, *merT*, *merP*, *merF* and *merR* and is closely related to the transposon Tn5053 of *Xanthomonas* sp. strain W17. Thus, it appears that the mercury-resistance transposons acquired by plasmids pB10 and pADP-1 are different. In addition, Mindlin *et al.* (2001) described various transposons carrying mercury-resistance determinants harboured by a diverse spectrum of environmental bacteria. The pB10 *mer* transposon is another element in this collection, again documenting the wide distribution of mercury-resistance determinants.

Resistance plasmids pB10 and R906 and the degradative plasmid pJP4 contain the same insertion of a Tn501-like transposon

Detailed analysis of the pB10 *mer* transposon insertion site revealed that pB10 possesses exactly the same insertion of a Tn501-like element in the same target site as identified previously in the IncP-1 β plasmids R906 and pJP4. The latter was found originally in *R. eutropha* and encodes degradation of 2,4-dichlorophenoxyacetic acid. The IR element downstream of the pB10 *merR* gene (IR_{*mer*}) is identical to the IR structure of the Tn501-like transposon inserted in both of the other plasmids (Smith & Thomas, 1987; Smith *et al.*, 1993). Sequence identity between these plasmids in this region extends beyond the IR elements and therefore it might be speculated that an element related to Tn501 transposed into a common ancestor. This conclusion is supported by the fact that the 5 bp direct repeat sequences adjacent to the IR_{*mer*} elements of all three plasmids are identical (TGCCT). Unfortunately, only 141 bps of the DNA sequence downstream of the replication gene *trfA1* are known for pJP4 but hybridization analyses showed that pJP4 contains a mercury-resistance region and the 3' end of the mercury regulatory gene *merR* is present on the sequenced fragment (Smith & Thomas, 1987; Burlage *et al.*, 1990). Fig. 4 shows the similarity between pB10 and pJP4 in this region. Recently published sequence data of pJP4 showed that this plasmid contains the mercury-resistance genes *merA*, *merD*, *merE* and *orf-2*, with the same organization as compared to pB10 (Clément *et al.*, 2001; GenBank accession no. AF225973). A 1700 bp region containing the pJP4 genes '*merA-merD-merE-orf2*' is 100% identical to the corresponding pB10 sequence, again supporting the close relationship of both plasmids.

The pJP4 *orf-2* was interrupted by insertion of an IS1071-like element (Clément *et al.*, 2001). An imperfect IR motif that is similar but not identical to the IR sequences of Tn501 and Tn21 was also found at the other end of the pB10 *mer* transposon. Therefore, it seems justified to designate the pB10 *mer* transposon 'Tn501-like', since it is not identical to Tn501 nor to Tn21. In addition to R906, which obviously contains a copy of this element, it appears that yet another IncP-1 β plasmid, namely R772, probably contains the same insertion of the Tn501-like transposon in the same target site as compared to pB10, R906 and pJP4 (Smith & Thomas, 1987). However, R772 mediates resistance only to kanamycin, and the IncP-1 β backbone has undergone an inversion. Therefore, the *mer* genes, remnants of which were detected by hybridization (Smith & Thomas, 1987), have clearly been inactivated in one way or another. The presence of exactly the same Tn501-like *mer* transposon insertion in pB10, R906, R772 and pJP4 may suggest either that these resistance and degradation plasmids have a common ancestor or that this common site is a hot-spot for insertion of transposable elements such as Tn501-like transposons.

Two more class II resistance transposons are inserted in the vicinity of the pB10 mercury-resistance region

Truncated derivatives of the streptomycin-resistance (*str*) transposon Tn5393c and the tetracycline-resistance (*tet*) transposon Tn1721 were also found in the pB10 'genetic load' region downstream of the replication initiation gene *trfA1* (Fig. 5).

Insertion of the *str* transposon Tn5393c disrupted the transposase gene *tnpA_{mer}* of the pB10 mercury-resistance transposon. A DNA stretch of 4364 bp containing a truncated *tnpA* gene (*tnpA_{str}*), the genes *tnpR*, *strA* and *strB* and an 81 bp IR structure (IR_{*strB*}) is 100 % identical to the Tn5393c sequence of the *Aeromonas salmonicida* plasmid pRAS2 (L'Abée-Lund & Sorum, 2000) and to Tn5393c present on the IncP-1 β plasmid pB4 (Tauch *et al.*, 2003). Truncation of the *tnpA_{str}* gene resulted from a transposition event of an IS1071-like element into this gene. A 110 bp IR structure which is identical to the IR elements of IS1071 was found at the insertion site of the pB10 IS1071-like element. The genes *strA* and *strB* of the pB10 *str* transposon encode the two different streptomycin-resistance proteins aminoglycoside-3"-phosphotransferase and aminoglycoside-6-phosphotransferase, respectively (Chiou & Jones, 1995). The IncP-1 β plasmid pB4 also contains a Tn5393c, but at a very different location in the plasmid than pB10, i.e. between the transfer genes *traM* and *traN* (*upf54.4*).

The second class II transposon in this pB10 region is a truncated derivative of the tetracycline-resistance transposon Tn1721, the insertion of which disrupted the *tnpA* gene of an IS1071-like element present on pB10 (see below). A 5407 bp stretch of DNA containing *tetR* encoding a

tetracycline-resistance repressor, *tetA* encoding a tetracycline efflux protein, a *pecM*-like gene and a truncated transposase gene (Δ *tnpA*) is 99.9 % identical to the corresponding DNA segment of the truncated *Escherichia coli* Tn1721 derivative integrated in pDEWT1 (GenBank accession no. AJ419171). The DNA region downstream of the pB10 *tetR* gene contains a second 5'-truncated *tnpA* gene which is nearly identical to the corresponding part of *tnpA* present on another *Escherichia coli* Tn1721 transposon (Allmeier *et al.*, 1992; GenBank accession no. X61367). The Tn1721-specific *tnpR* gene encoding a resolvase and *orfI* which is also an integral part of this transposon are missing in the pB10 sequence. The *pecM*-like gene located downstream of *tetA* is also present in the published Tn1721 sequences but has not been annotated until now. The encoded gene product is 61 % identical to the PecM regulator protein of *Agrobacterium tumefaciens* C58 (GenBank accession no. NP_530979) and 41 % identical to PecM of the phytopathogenic bacterium *Erwinia chrysanthemi* (GenBank accession no. X74409). Two copies of the Pfam motif PF00892 (DUF6) characteristic for hypothetical membrane proteins of unknown function were found in the pB10 PecM-like sequence. The *Erwinia chrysanthemi* PecM protein is an integral membrane protein and it was suggested that it is involved in the perception and/or transduction of an external environmental signal leading to a modulation of pectinase gene expression (Reverchon *et al.*, 1994). The function of the *pecM*-like gene on Tn1721 remains unknown. Finally, two IR sequences were found in the pB10 Tn1721-like element that overlap the stop codons of the truncated *tnpA* genes and are identical to the IRRII structure of the truncated Tn1721 derivative of *Escherichia coli* (GenBank accession no. AJ419171).

The pB10 insertion element IS1071 is not associated with degradative genes

Plasmid pB10 is the first completely sequenced resistance plasmid reported to contain the insertion sequence element IS1071 (Fig. 5), which has so far only been found to flank several degradative genes [Tralau *et al.*, 2001; Martinez *et al.*, 2001; Rousseaux *et al.*, 2002; Clément *et al.*, 2001; Boon *et al.*, 2001; Nakatsu *et al.*, 1991; for an overview, see di Gioia *et al.* (1998)]. Insertion of the IS1071 element on pB10 disrupted the *tnpA* gene of Tn5393c and the IS1071-specific *tnpA* gene itself was split by insertion of the Tn1721 derivative. The pB10 IS1071 element, which is flanked by two 110 bps IR elements, is almost identical to the IS1071 elements found on the *Comamonas testosteroni* plasmid pTSA (Tralau *et al.*, 2001) and on the degradative plasmid pADP-1 (Martinez *et al.*, 2001). A *tnpA_{IS1071}* segment encoding the 135 internal amino acids of TnpA is missing in the pB10 sequence, which might be explained by a deletion event that occurred during or after transposition of the Tn1721 derivative.

Copies of IS1071 were found on other IncP-1 β plasmids and on plasmids carrying degradative genes. The atrazine

catabolic plasmid pADP-1 contains three complete copies of the *IS1071* element. Two of them flank a region containing the *atzA* gene which encodes the degradative enzyme atrazine chlorohydrolase. The third copy is linked to *atzB* for hydroxyatrazine hydrolase and to the *mer* region of this plasmid (Martinez *et al.*, 2001). Rousseaux *et al.* (2002) demonstrated a strong correlation between the presence of *IS1071* and genes involved in atrazine degradation for plasmids present in *Chelatobacter* and *Arthrobacter* strains. The *tsa* genes for *p*-toluenesulfonate degradation located on the IncP-1 β plasmid pTSA are organized on a composite transposon flanked by two *IS1071* elements. An analogous structure was found for the IncP-1 β plasmid pJP4 responsible for the catabolism of 2,4-dichlorophenoxyacetate and 3-chlorobenzoate, where the degradative *tfd* genes are framed by *IS1071* and an *IS1071*-like element (Clément *et al.*, 2001). Likewise, Boon *et al.* (2001) reported the linkage of plasmid-borne *tdnQ* genes involved in the oxidative deamination of aniline to the *IS1071* element and suggested that this insertion sequence element might be involved in the dissemination of aniline degradation genes in the environment.

As far as we know, pB10 and R906 are the only IncP-1 β plasmids known so far that contain a copy of *IS1071* that is not associated with any degradative genes.

In summary, the 19.3 kb 'genetic load' region between *oriV* and the replication initiation gene *trfA1* of pB10 is composed of four truncated class II (Tn3 family) transposable elements (Tn501-like, Tn5393c, Tn1721 and *IS1071*). None of the transposable elements contain an intact transposase gene and consequently the organization of these elements on pB10 cannot be rearranged by the action of pB10-encoded transposases. Furthermore, comparison with other IncP-1 β plasmids confirms previously made observations that the region downstream of the *trfA1* gene is a hot-spot for insertions of transposable elements (Smith & Thomas, 1987; Thorsted *et al.*, 1998).

Evolution of the resistance plasmid pB10

The evolutionary history of pB10 can be explained by different scenarios, some of which are suggested below. (i) An ancestral degradative plasmid related to pADP-1 or pTSA has lost its degradation genes by a deletion event, and different transposons carrying resistance genes were subsequently integrated. This hypothesis is supported by the fact that pB10 contains only one *IS1071* element. As mentioned before, the degradative genes of pTSA, pADP-1 and pJP4 are flanked by at least two copies of *IS1071*. Deletion of degradative genes framed by *IS1071* elements in the same orientation might occur by a recombination event between the two IS sequences. Moreover, so far as we know, *IS1071* has only been reported to flank degradative genes, and not antibiotic-resistance genes. (ii) An ancestral plasmid carrying a Tn501-like mercury-resistance transposon, probably also the ancestor of plasmids pJP4, R906 and R772 (Smith & Thomas, 1987), might have

evolved towards plasmid pB10 by integration of different resistance transposons.

Another event in the evolution of pB10 would then be an exchange of part of the central regulation/stability region with a corresponding segment that shows high similarity to that of pB4, an antibiotic-resistance IncP-1 β plasmid isolated from the same waste-water treatment plant. Plasmid pB10 is the first clear example of an IncP-1 plasmid with this kind of a mosaic structure of the backbone (shown in Fig. 1). Although highly speculative, it may be possible that the ancestor of pB10, whose entire backbone was very similar to that of the R751/pADP-1/pTSA group, recombined with a plasmid similar to pB4 in the activated sludge community of the waste-water treatment plant. Such recombination would require temporary co-existence of two incompatible plasmids within one cell, which seems very well possible so long as the two plasmids have different accessory genes that are both under selection. Multiple parallel selection is easy to envisage in a waste-water treatment plant since the incoming water can typically contain a large variety of compounds, such as various pesticides and antimicrobial agents, some of which may select for plasmid-encoded genes. Extensive recombination between closely related plasmids, although rather contrary to the idea of surface exclusion being a barrier to entry of related plasmids, has been indicated by studies on F-like plasmids (Boyd *et al.*, 1996). If such a situation is common in many plasmid groups in natural environments, it indicates an additional route to rapid evolution of plasmid-borne traits. Whether the recombination took place recently in the activated sludge basin, or at some much more distant time point, should become clear by more extensive sequencing of selected parts of the R906 backbone to determine whether the same mosaic pattern existed as long ago as the 1970s.

In conclusion, several observations point towards a close relationship of pB10 (and R906) to a few known degradative IncP-1 β plasmids. (i) Three backbone modules show the highest degree of similarity to the corresponding modules of the degradative IncP-1 β plasmids pADP-1 and pTSA. (ii) Plasmid pB10 seems to be evolutionarily related to the degradative plasmid pJP4 since both plasmids possess the same Tn501-like mercury-resistance transposon inserted in the same target site. (iii) Plasmid pB10 harbours a copy of *IS1071*, which was frequently found to be associated with degradative genes. These observations confirm the notion that IncP-1 plasmids should be seen as shuttles able to exchange temporarily useful genes between bacterial populations in different environments (Smalla & Sobczyk, 2002; Top *et al.*, 2002; Turner *et al.*, 2002). They consist of large 'backbones' that may pick up and later lose different kinds of accessory genes. The same backbones can thus carry antibiotic-resistance determinants at one point, and degradative operons later on, or *vice versa*, and possibly both if they are both selected for. The kind of genetic load (resistance or xenobiotic metabolism) that is found on

an isolated plasmid would thus depend on the selective pressure its host has encountered most recently, which in turn would be determined by the environment the plasmid has resided in before it was isolated. As far as we know, the IncP-1 plasmids characterized so far carry either antibiotic-resistance genes or degradative genes, but never both. Considering the sequences already available from previous studies, as well as the new sequence information of pB10, it seems unlikely that these two groups of IncP-1 β plasmids ('antibiotic-resistance' and 'degradative') have evolved as separate lineages. Moreover, the sequence of pB4 clearly revealed that not all IncP-1 β resistance plasmids evolved from one ancestor, since the backbone regions of the resistance plasmid pB4 are only distantly related to those of the other IncP-1 β plasmids. A more plausible explanation for the lack of isolated IncP-1 β plasmids with both resistance and degradation genes is the fact that these plasmids were isolated from environments that had been exposed long enough to only one of the two selective pressures – either an organic pollutant (in soils, sludges, water, etc.) or antibiotics (in clinical environments) – such that the predominant plasmids only retained one of those two sets of accessory genes. Although the limited number of complete IncP-1 plasmid sequences makes these hypotheses still highly speculative, the sequence of pB10 gives a few new hints as to the possible evolutionary history of these highly transferable and very widespread plasmids. Apparently, the IncP-1 β -specific backbone modules for replication, maintenance, stable inheritance and conjugative transfer, which in the case of pB10 were derived from two distinct ancestral IncP-1 β plasmids, are used for an efficient dissemination of genes that code for various resistances and for degradation of xenobiotics. Therefore, such plasmids must play a major role in rapid adaptation of bacterial communities to changing environments.

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