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ORIGINAL PAPER

A. Tauch · A. Schlüter · N. Bischoff · A. Goesmann
F. Meyer · A. Pühler

The 79,370-bp conjugative plasmid pB4 consists of an IncP-1β backbone loaded with a chromate resistance transposon, the *strA-strB* streptomycin resistance gene pair, the oxacillinase gene bla_{NPS-1}, and a tripartite antibiotic efflux system of the resistance-nodulation-division family

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Abstract Plasmid pB4 is a conjugative antibiotic resistance plasmid, originally isolated from a microbial community growing in activated sludge, by means of an exogenous isolation method with *Pseudomonas* sp. B13 as recipient. We have determined the complete nucleotide sequence of pB4. The plasmid is 79,370 bp long and contains at least 81 complete coding regions. A suite of coding regions predicted to be involved in plasmid replication, plasmid maintenance, and conjugative transfer revealed significant similarity to the IncP-1β backbone of R751. Four resistance gene regions comprising mobile genetic elements are inserted in the IncP-1β backbone of pB4. The modular 'gene load' of pB4 includes (1) the novel transposon Tn*5719* containing genes characteristic of chromate resistance determinants, (2) the transposon Tn*5393c* carrying the widespread streptomycin resistance gene pair *strA-strB*, (3) the β-lactam antibiotic resistance gene bla_{NPS-1} flanked by highly conserved sequences characteristic of integrons, and (4) a tripartite antibiotic resistance determinant comprising an efflux protein of the resistance-nodulation-division (RND) family, a periplasmic membrane fusion protein (MFP), and an outer membrane factor (OMF). The components of the RND-MFP-OMF efflux system showed the highest similarity to the products of the *mexCD-oprJ* determinant from the *Pseudomonas aeruginosa* chromosome. Functional analysis of the cloned resistance region from pB4 in *Pseudomonas* zsp. B13 indicated that the RND-MFP-OMF efflux system conferred high-level resistance to erythromycin and roxithromycin resistance on the host strain. This is the first example of an RND-MFP-OMF-type antibiotic resistance determinant to be found in a plasmid genome. The global genetic organization of pB4 implies that its gene load might be disseminated between bacteria in different habitats by the combined action of the conjugation apparatus and the mobility of its component elements.

Keywords IncP-1β plasmid · Horizontal gene transfer · Plasmid evolution · Resistance-nodulation-division family · Multidrug efflux system

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A. Tauch (✉) · N. Bischoff · A. Goesmann · F. Meyer
Zentrum für Genomforschung, Universität Bielefeld,
Universitätsstrasse 25, 33615 Bielefeld, Germany
E-mail: Andreas.Tauch@genetik.uni-bielefeld.de
Tel.: +49-521-1064823
Fax: +49-521-1065626

A. Schlüter · A. Pühler
Lehrstuhl für Genetik, Universität Bielefeld,
Universitätsstrasse 25, 33615 Bielefeld, Germany

Introduction

During the past decade, bacteria that cause human disease have developed resistance to many antibiotics commonly used for treatment (Tan et al. 2000). This rise in the antibiotic resistance of human pathogens is a growing medical problem in nearly every infectious disease. The rapid development of drug resistance in bacterial pathogens has been attributed in part to the inappropriate use of antimicrobials in human and veterinary medicine and in agriculture (Witte 1998; Tan et al. 2000; Teuber 2001). Resistance to antibiotics can arise either from point mutations in the bacterial genome (intrinsic resistance) or through the acquisition of genes encoding resistance determinants (acquired resistance). Broad-host-range conjugation is thought to be the main mechanism by which plasmid-encoded antibiotic resistance genes are transferred between bacteria (Tan et al. 2000). In addition, antibiotic resistance genes have become highly mobile since the development of antimicrobial chemotherapy (Séveno et al. 2002). Conjugative plasmids can carry different types of transposons and integrons with

multiple resistance genes or multiple gene cassettes, and can disseminate this 'gene load' widely by conjugation. Mobile gene cassettes and resistance genes located on transposons can then be integrated into the chromosome of the bacterial recipient or they can be maintained in an extrachromosomal state on the plasmid. The increasing appearance of large multidrug resistance plasmids is also a direct result of strong selective pressure resulting from antimicrobial chemotherapy (Séveno et al. 2002). Several examples of this medically alarming development have been inferred from the complete nucleotide sequences of plasmid genomes. Thus, multidrug resistance plasmids from *Lactococcus lactis* (Perreten et al. 1997), *Staphylococcus aureus* (Berg et al. 1998), *Corynebacterium striatum* (Tauch et al. 2000), and *Enterococcus faecalis* (Schwarz et al. 2001) show a modular genome organization with distinct genetic segments representing antibiotic resistance determinants. These DNA sequence data provide molecular evidence that natural routes exist by which antibiotic resistance determinants from bacteria that occupy different habitats can be assembled in a single plasmid genome. Therefore, analyses of the reservoirs of antibiotic resistance determinants are of great importance for improving our understanding of the molecular evolution of multidrug resistance plasmids and the transfer of antibiotic resistance genes into human pathogens.

The application of molecular detection techniques in studies of microbial ecology now allows detailed investigation of the reservoirs of antibiotic resistance genes (Dröge et al. 1999). Recently, microbial communities found in activated sludge from a municipal sewage treatment plant were characterized by an exogenous plasmid isolation method, with a green-fluorescent derivative of *Pseudomonas* sp. B13 serving as the recipient strain in filter matings (Dröge et al. 2000). A total of twelve distinct plasmids with sizes ranging from 41 to 69 kb were identified in *Pseudomonas* sp. B13. As deduced from replicon typing, most of the plasmids belonged to the IncP incompatibility group and were partly characterized as broad-host-range plasmids with extremely high transfer frequencies in filter matings. In addition, antibiotic susceptibility screening revealed that the plasmids conferred various patterns of antibiotic resistance on their hosts. For instance, the newly identified plasmid pB4 (with a calculated size of approximately 69 kb) conferred resistance to the antibiotics erythromycin, streptomycin, and amoxicillin in *Pseudomonas* sp. B13 (Dröge et al. 2000). Remarkably, the amino acid sequence deduced from a single terminal sequence read of a cloned restriction fragment of pB4 displayed similarity to multidrug efflux systems of the resistance-nodulation-division (RND) family (Dröge et al. 2000). In gram-negative microorganisms, RND multidrug transporters work in conjunction with a periplasmic membrane fusion protein (MFP) and an outer membrane protein, termed outer membrane factor (OMF), which together facilitate the passage of a variety of substrates into the external medium (Zgurskaya and Nikaido 2000). Such tripartite multidrug efflux systems have been described in a number of organisms and are typically encoded on the chromosome (Poole 2001a). In *Pseudomomas aeruginosa* four structurally related RND-MFP-OMF-type multidrug resistance determinants have been investigated to date (Poole 2001b), and six additional systems have been predicted from the complete genome sequence of *P. aeruginosa* PAO1 (Stover et al. 2000). Most RND efflux pumps possess broad substrate specificity and confer resistance to a wide range of clinically relevant antibiotics (Poole 2001a). Thus, the presence of a multidrug resistance determinant of the RND-MFP-OMF-type on a conjugative IncP plasmid implies frightening scenarios with regard to its dissemination into human pathogens by horizontal gene transfer. Therefore, we decided to determine the complete nucleotide sequence of the resistance plasmid pB4 and explore its molecular genetic organization.

In this paper, we present the detailed annotation of the nucleotide sequence of the resistance plasmid pB4, which yields insights into its global genetic organization. By computer-assisted DNA sequence analysis we have identified the genetic determinants involved in mediating antibiotic resistance in *Pseudomonas* sp. B13. These resistance determinants are part of four distinct plasmid segments which were obviously inserted into an IncP-1β plasmid backbone during plasmid evolution. The sequencing and annotation of pB4 provides a novel example of the modular evolution of plasmid genomes and demonstrates how horizontal mobile elements contribute to the rapid spread of antibiotic resistance determinants into human pathogens.

Materials and methods

Bacterial strains and growth conditions

Plasmid pB4 was originally obtained in a green fluorescent derivative of *Pseudomonas* sp. B13 by an exogenous plasmid isolation method, and was subsequently transferred to *Escherichia coli* DH5αMCR (Dröge et al. 2000). *E. coli* DH5αMCR carrying pB4 was grown in Antibiotic Medium No. 3 (Oxoid, Wesel, Germany) supplemented with 12 μg/ml streptomycin. *Pseudomonas* sp. B13 and *Sinorhizobium meliloti* FP2 (Schneiker et al. 2001) were routinely grown at 30°C in Luria-Bertani (LB) medium and in TY complex medium, respectively (Sambrook et al. 1989; Beringer 1974). Recombinant plasmids were selected for by using kanamycin (50 μg/ml) for pZErO-2 derivatives and tetracycline (5 μg/ml) for pJP2 derivatives.

Recombinant DNA techniques and construction of recombinant plasmids

Vector DNA and recombinant plasmids were extracted from *E. coli* by means of the QIAprep Spin Miniprep kit (Qiagen, Hilden, Germany). All cloning experiments were performed in *E. coli* TOP10 (Invitrogen, Karlsruhe, Germany). Recombinant DNA techniques followed standard procedures (Sambrook et al. 1989). Transfer of recombinant plasmids from *E. coli* to *Pseudomonas* sp. B13 and *S. meliloti* FP2 was performed by filter matings as described previously (Dröge et al. 2000). *E. coli* S17-1 was used as the mobilizing donor strain (Simon et al. 1983).

To perform functional analyses of antibiotic resistance genes from pB4, the bla_{NPS-1} gene and the *nfxB-mexCD-oprJ* determinant were cloned in *E. coli* TOP10. A 1.6-kb *Bam*HI fragment of pB4 carrying the bla_{NPS-1} gene was cloned into the *Bam*HI site of the vector pZErO-2 (Invitrogen), resulting in pZErO-bla_{NPS-1}. In addition, a 6.9-kb *Spe*I-*Nhe*I DNA fragment carrying the predicted resistance determinant *mexCD-oprJ* and the putative regulatory gene *nfxB* was cloned into the *Spe*I site of pZErO-2. The resulting plasmid was termed pZErO-*mex*. An *nfxB* deletion derivative of pZErO-*mex*, designated pZErO-Δ*nfxB*, was subsequently constructed by deleting a 0.5-kb *Spe*I-*Sac*II DNA fragment comprising the 3′end of the *nfxB* gene. Both the complete resistance determinant and the deletion derivative were cloned as *Bam*HI-*Xba*I fragments into the broad-host-range mini-RK2 derivative pJP2 (Prell et al. 2000). The resulting constructs, designated pJP2-*mex* and pJP2-Δ*nfxB*, were finally transferred to *Pseudomonas* sp. B13 and *S. meliloti* FP2 by conjugation.

Library construction and DNA sequencing of pB4

pB4 DNA was isolated from *E. coli* DH5αMCR by alkaline-SDS lysis and column purification according to the NucleoBond plasmid purification protocol (Macherey-Nagel, Düren, Germany). A randomly sheared insert shotgun library of pB4 was constructed in *E. coli* TOP10 by cloning 1.3–1.5-kb size fractions of the plasmid into the vector pCR-Blunt II-TOPO (GATC, Konstanz, Germany). Templates for DNA sequencing were prepared from *E. coli* clones carrying recombinant plasmids by automated alkaline lysis with the BioRobot 9600 (Qiagen). Purified template DNA was sequenced by cycle-sequencing reactions on a Prism ABI 377 DNA sequencer using the Prism Ready Reaction Dye Deoxy Termination kit and fluorescently labeled universal and reverse primers. Computer-assisted assembly of the random shotgun sequences was carried out with the gap4 program from the STADEN sequence analysis package (Staden 1996). Gaps in the pB4 plasmid sequence were closed by primer walking with oligonucleotides designed on the basis of contig ends (Staden 1996) using pB4 DNA as template. Final assembly and editing of the DNA sequence data resulted in a single, circular molecule with a total length of 79,370 bp.

DNA sequence analysis and annotation

After nucleotide sequence assembly with the Staden (1996) package, the sequence contig file was uploaded to the GenDB system (Zentrum für Genomforschung, Bielefeld, Germany). Here, after gene prediction with GLIMMER 2.0 (Delcher et al. 1999), the resulting ORFs were manually annotated using evidence from BLAST analysis (Altschul et al. 1997) against the EMBL nucleotide sequence database. In addition, the complete sequence of pB4 was searched for σ^{70}-dependent promoters using the Neural Network Promoter Prediction approach (Reese et al. 1996). Repeat regions within the pB4 nucleotide sequence were identified with the REPuter software (Kurtz et al. 2001). Global amino acid sequence similarities were calculated with the computer program ALIGN (Myers and Miller 1988). Helix-turn-helix DNA-binding motifs were detected with the algorithm of Dodd and Egan (1990). Prediction of transmembrane helical segments was performed with the neural network system PredictProtein (Rost et al. 1995). Finally, the annotated DNA sequence of pB4 was reoriented in such a way that the orientation and the order of genes is the same as in the IncP-1β prototype plasmid R751 (Thorsted et al. 1998). The annotated sequence of pB4 is available in the EMBL database under the Accession No. AJ431260.

Antibiotic resistance assays

Initial susceptibility testing of *Pseudomonas* sp. B13 carrying pJP2, pJP2-*mex* and pJP2-Δ*nfxB* was performed by a disk diffusion assay on Iso-Sensitest Agar (Oxoid) with antibiotic disks supplied by Oxoid, containing amoxicillin (AML25), amoxicillin and clavulanic acid (AMC30), aztreonam (ATM30), cefaclor (CEC30), cefazolin (KZ30), cefepime (FEP30), cefotaxime (CTX30), cefoxitin (FOX30), cefpirome (CPO30), ceftazidime (CAZ30), cefuroxime (CXM30), chloramphenicol (C10), ciprofloxacin (CIP5), erythromycin (E30), gentamicin (CN30), imipenem (IMP10), norfloxacin (NOR5), oxacillin (OX5), piperacillin (PRL30), rifampicin (RD30), and tetracycline (TE10). The activity of β-lactam antibiotics against *E. coli* TOP10 carrying pZErO-bla_{NPS-1} was quantified by determination of the minimum inhibitory concentration (MIC) using a macrobroth dilution method recommended by the National Committee of Clinical Laboratory Standards (1997). The cloned RND-MFP-OMF gene region of pB4 was further analyzed in *Pseudomonas* sp. B13, *E. coli* S17-1, and *S. meliloti* FP2 by the macrobroth dilution method. Antibiotics for susceptibility testing were purchased from Sigma-Aldrich (Taufkirchen, Germany) and ICN Biomedicals (Eschwege, Germany).

Results and discussion

The resistance plasmid pB4 shows the genetic organization characteristic of an IncP-1β type plasmid, and is loaded with four distinct resistance gene regions

The complete nucleotide sequence of the resistance plasmid pB4 was established by terminal sequencing of inserts from a randomly-sheared shotgun library and subsequent primer walking strategies. The nucleotide sequence was determined on both strands with a mean coverage of 6.9 per consensus base, resulting in a circular DNA sequence of 79,370 bp with an overall G + C content of 62.6%. pB4 is thus 10 kb larger than the previous size estimation on the basis of mapping with the restriction enzyme *Not*I (Dröge et al. 2000). The pB4 sequence contains 81 complete coding regions and two pseudogenes (3′ends of *orf30* and *parA*), as identified by GLIMMER 2.0 and manual annotation with GenDB. A summary of the sequence data for pB4 including the position and G + C content of the coding regions, the length and molecular mass of the predicted proteins, and the best homologies to known proteins in databases are summarized in Table 1. Figure 1 presents a detailed map of the genes found on pB4 as well as indicating the approximate positions of additional genetic features.

The predicted coding regions of pB4 are dominated by genes involved in plasmid replication, plasmid partitioning, plasmid maintenance, and conjugative transfer, while a second set of coding regions can be attributed to the mobile 'gene load' which might be involved in resistance functions (Table 1, Fig. 1). The global gene organization of pB4 was found to be highly similar to the genetic structure of the IncP-1β plasmid R751 (Table 1; Thorsted et al. 1998). This observation is compatible with previous replicon typing of pB4 by means of *trfA2*-specific primers (Dröge et al. 2000). However, some differences were observed upon detailed comparison of the pB4 and R751 plasmid backbones. In particular, two small genes (*kleB* and *kleG*) located in a plasmid region of R751 devoted to stable inheritance (Thorsted et al. 1998) were not identified on pB4 (Fig. 2). On the other hand, two hypothetical coding

regions of unknown function (*upfA* and *upfB*) and the remnant (3′end) of a *parA* -like gene were identified upstream of the β-lactam antibiotic resistance region (Figs. 1 and 2). Partitioning (Par) proteins are a rather characteristic element of IncP-1α plasmids and are proposed to play an important role in inheritance by multimer resolution, killing of plasmid-free segregants and possibly by active partitioning (Pansegrau et al. 1994; Thorsted et al. 1998). Differences between the pB4 and R751 backbones are thus present in a distinct DNA region encoding plasmid inheritance functions and in a DNA region of unknown function (Fig. 2; Thorsted et al. 1998). Since all the genes potentially related to the IncP-1β backbone have been identified on pB4, comparative and experimental studies of the respective proteins in IncP-1β plasmids will eventually facilitate the full understanding of their molecular function.

An automated search for σ^{70}-dependent promoters resulted in the prediction of a set of putative promoter sequences when a cutoff score of 0.80 was used in the Neural Network Promoter Prediction program (Reese et al. 1996). Following the logic of the predicted gene organization of pB4, we screened the candidates for those located upstream of the first coding region in each operon. Additional promoters upstream of other coding regions were chosen if their position relative to the ribosome-binding site seemed logical. A total of 20 putative pB4 promoter sequences were selected on the basis of these criteria (Fig. 1, Table 2). Additional structural features of the IncP-1β plasmid backbone such as KorA (Table 3), KorB (Table 4), and KorC operator sequences (Table 5) were identified in the pB4 plasmid sequence. The KorABC proteins of R751 are involved in the coordinate regulation of replication and transfer functions (Thorsted et al. 1998). In contrast to the KorA and KorB operator sequences, the binding-sites for KorC differ substantially between pB4 and R751 (Table 5). Furthermore, a set of conserved inverted repeats of unknown function first noted in R751 (Thorsted et al. 1998) was detected in the pB4 genome (Table 6). These sequence data suggest that not only the general genetic organization but also the patterns of gene expression and regulation of pB4 are very similar to those deduced from the R751 plasmid genome.

Gene regions specifying the 'gene load' of R751, namely the mobile elements Tn*4321* and Tn*402*/*5090*, are absent from the pB4 genome, with the exception of the *tniC* gene which is known from ancestral transposition modules of integrons (Fig. 2 and Table 1). Interestingly, parts of the specific 'gene load' of pB4 are located at similar positions to the mobile elements of R751 (Fig. 2). The insertions found in the pB4 plasmid are thus located at the junctions between sectors of IncP-1β specifying different molecular functions such as plasmid maintenance and stability, plasmid replication, and conjugative transfer (Fig. 2). These insertion sites are obviously not critical for the proper functioning of the IncP-1β plasmid backbone (Thorsted et al. 1998). The DNA regions representing the specific 'gene load' of pB4 will be discussed in more detail below.

The novel class II transposon Tn*5719* carries a gene set characteristic of chromate resistance determinants

Annotation of the complete pB4 nucleotide sequence revealed that a novel class II Tn*501*-type transposon, termed Tn*5719*, was inserted into the IncP-1β plasmid backbone upstream of the conserved inverted repeat sequence IR5 (Fig. 1). The presence of a 5-bp target site duplication indicated that transposon Tn*5719* had obviously integrated into pB4 by a single transpositional insertion. Interestingly, the DNA regions flanking Tn*5719* showed significant sequence similarity to conserved 38-bp terminal inverted repeats associated with transposons of the Tn*3* family. The presence of an additional 5-bp direct repeat flanking these transposon termini suggests that Tn*5719* integrated into another class II transposon, the central coding regions of which were deleted during plasmid evolution (Fig. 3). This nucleotide sequence annotation supports the conclusion of Thorsted et al. (1998) that the *klcA*-*trfA* intergenic region represents a hotspot for the insertion of transposons into the IncP-1β plasmid backbone. This hypothesis is further supported by the observation that another Tn*3*-like transposase gene (*tnpA2*) is located adjacent to Tn*5719* and might be responsible for an additional 'gene load' of pB4 at this site (Fig. 1 and Table 1). Likewise, complex insertion events have been associated with transposon Tn*4321* in R751 (Thorsted et al. 1998).

Transposon Tn*5719* has a length of 8355 bp and contains seven coding regions, including two conserved genes (*tnpR* and *tnpA*) which appear to encode the transposase and resolvase functions of the mobile element (Fig. 3 and Table 1). The coding regions of *chrB* and *chrA*, as well as those of the adjacent genes *chrC*, *orf44*, and *orf46*, are organized in such a way that the start and stop codons overlap, indicating translational coupling of the respective genes. The deduced gene products of *chrB* and *chrA* revealed significant similarity to plasmid-encoded chromate resistance determinants (Table 1), suggesting that Tn*5719* might be involved in resistance to the transition metal chromium, which has become a serious pollutant in diverse environmental settings due to its widespread industrial use (Cervantes et al. 2001). Chromium is a highly toxic, non-essential, metal for microorganisms and exists mainly in the oxyanion form (i.e. CrO_4^{2-}). The putative chromate resistance determinant of pB4 was thus compared with chromate resistance regions previously identified on the *P. aeruginosa* plasmid pUM505 and on the *Ralstonia metallidurans* plasmid pMOL28 (Fig. 3). Global amino acid sequence similarity was observed to the membrane protein ChrA and the ChrB protein of pMOL28, which have been shown to be essential for an inducible chromate resistance (Nies et al. 1990). The ChrB protein was assigned an additional role in the regulation and inducibility by chromate of the chromate resistance determinant (Nies et al. 1990). On the other hand, the single ChrA protein of pUM505 was sufficient to determine a constitutive chromate resistance in *P. aeru-*

Table 1 Location of the predicted coding regions of pB4 andclosest relationship of the deduced proteins

Coding sequence	pB4 co-ordinates (5′ → 3′)	G + C content (%)	Protein			Degree of sequence identity/similarity to best homolog; source[a]	GenBank No.
			Number of residues	Molecular mass (kDa)	pI		
upf54.4	1480–158	64.1	440	48.2	6.30	33%/39% to Upf54.4; plasmid R751	U67194
upf54.8	1871–1524	54.3	115	12.7	10.21	79%/87% to Upf54.8; plasmid R751	U67194
kfrA	2984–2034	69.8	316	33.6	4.85	67%/77% to KfrA; plasmid R751	U67194
korB	4213–3152	65.4	353	38.7	4.82	88%/90% to KorB; plasmid R751	U67194
incC2	4974–4210	64.7	254	27.5	7.47	88%/92% to IncC2; plasmid R751	U67194
incC1	5292–4210	64.2	360	38.0	9.31	84%/89% to IncC1; plasmid R751	U67194
korA	5279–4971	62.8	102	12.1	9.71	89%/95% to KorA; plasmid R751	U67194
kleF	5715–5386	64.0	109	10.4	4.13	77%/82% to KleF; plasmid R751	U67194
kleE	6043–5717	63.3	108	11.9	9.70	83%/88% to KleE; plasmid R751	U67194
kleA	6488–6252	67.9	78	8.7	4.87	69%/73% to KleA; plasmid R751	U67194
korC	6902–6645	63.2	85	9.4	5.15	78%/85% to KorC; plasmid R751	U67194
klcB	8121–6919	69.7	400	43.6	6.46	81%/87% to KlcB; plasmid R751	U67194
klcA	8824–8396	66.9	142	15.6	4.34	93%/96% to KlcA; plasmid R751	U67194
din	8964–9260	62.3	98	10.6	5.61	66%/73% to DNA-damage-inducible protein XF2081; *Xylella fastidiosa*	
nfxB	10005–9445	56.0	186	20.5	5.73	61%/71% to transcriptional regulator NfxB; *Pseudomonas aeruginosa*	AE004874
mexC	10183–11349	63.1	388	36.3	7.31	66/78% to RND multidrug efflux membrane fusion protein MexC precursor; *Pseudomonas aeruginosa*	AE004873
mexD	11364–14507	61.3	1047	112.3	4.81	80%/87% to RND multidrug efflux transporter MexD; *Pseudomonas aeruginosa*	AE004873
oprJ	14513–15940	64.2	475	51.3	5.04	67%/79% to outer membrane protein OprJ precursor; *Pseudomonas aeruginosa*	AE004873
orf19	17798–16740	57.8	352	40.2	7.81	–	–
istA	18306–19865	59.0	519	60.0	9.81	44%/54% to IS *S12* transposase subunit 1; *Pseudomonas putida*	AF292393
istB	19859–20593	57.6	244	27.4	9.61	50%/65% to IS *S12* transposase subunit 2; *Pseudomonas putida*	AF292393
orf24	20912–20655	52.7	85	9.4	5.24	–	–
orf25	21573–21034	65.6	179	19.7	12.01	–	–
orf26	22390–21815	67.2	191	20.4	11.87	–	–
orf28	22509–22955	56.2	148	15.6	4.81	–	–
invA	23542–22985	56.5	185	20.4	10.32	90%/95% to DNA-invertase; *Rhodospirillum rubrum* plasmid pKY1	D17434
orf30[b]	23791–23536	56.6	84	9.7	4.87	38%/57% to hypothetical protein AGR_C_4225AP; *Agrobacterium tumefaciens*	AE008147
orf32	24014–24838	51.6	274	30.4	5.07	–	–
orf33	25031–25618	53.6	195	20.3	6.32	–	–
orf36	25663–26109	56.2	148	15.6	4.81	–	–
invB	26696–26139	56.5	185	20.4	10.32	90%/95% to DNA-invertase; *Rhodospirillum rubrum* plasmid pKY1	D17434
orf38	27082–26690	55.7	130	14.9	4.42	38%/57% to hypothetical protein AGR_C_4225AP; *Agrobacterium tumefaciens*	AE008147
orf40	27369–27079	59.1	96	12.6	7.28	64%73% to hypothetical protein AGR_C_4229p; *Agrobacterium tumefaciens*	AE008148
tnpA2	27530–30496	62.9	988	105.1	9.07	89%/92% to Tn*21* transposase; *Shigella flexneri* plasmid R100	AP000342
orf44	31900–30686	67.6	404	42.7	9.67	58%/70% to putative transport transmembrane protein SMc00807; *Sinorhizobium meliloti*	AL591784
orf46	32355–31900	65.6	151	16.7	5.71	48%/60% to hypothetical protein Mlr5406; *Mesorhizobium loti*	AP003006
chrC	32951–32352	66.2	199	21.4	4.88	72%/78% to ChrC protein; *Ralstonia metallidurans* plasmid pMOL28	AJ313327
chrA	34243–33011	65.1	410	43.4	9.75	93%/96% to chromate efflux protein ChrA; *Pseudomonas aeruginosa* plasmidpUM505	M29034
chrB	35184–34240	67.6	314	34.3	4.82	46%/59% to ChrB protein; *Ralstonia metallidurans* plasmid pMOL28	AJ313327
tnpR	35408–35968	66.1	186	21.4	10.43	81%/89% to Tn*21* resolvase; *Shigella flexneri* plasmid R100	AP000342
tnpA	35971–38940	64.9	989	111.7	8.66	66%/77% to Tn*21* transposase; *Shigella flexneri* plasmid R100	AP000342
trfA2	40294–39425	63.0	289	33.5	9.03	84%/89% to S-TrfA; plasmid R751	U67194
trfA1	40648–39425	66.2	407	45.9	9.38	75%/81% to TrfA1; plasmid R751	U67194
ssb	41035–40697	58.7	112	12.8	7.42	89%/90% to Ssb; plasmid R751	U67194
trbA	41145–41504	59.7	119	13.7	10.06	91%/96% to TrbA; plasmid R751	U67194

Table 1 (Contd.)

Coding sequence	pB4co-ordinates (5′ → 3′)	G + C content (%)	Protein			Degree of sequence identity/similarity to best homolog; source[a]	GenBank No.
			Number of residues	Molecular mass (kDa)	pI		
trbB	41819–42784	62.2	321	36.9	7.79	93%96% to TrbB; plasmid R751	U67194
trbC	42798–43262	65.6	154	16.3	6.33	89%/93% to TrbC; plasmid R751	U67194
trbD	43266–43577	62.5	103	11.9	11.60	95%/97% to TrbD; plasmid R751	U67194
trbE	43574–46132	63.8	852	94.1	5.97	92%/96% toTrbE; plasmid R751	U67194
trbF	46129–46917	64.6	262	29.0	9.63	82%/90% to TrbF; plasmid R751	U67194
trbG	46914–47828	63.4	304	33.6	9.31	84%/90% to TrbG; plasmid R751	U67194
trbH	47831–48292	68.4	153	16.7	9.24	76%/85% to TrbH; plasmid R751	U67194
trbI	48296–49678	66.5	460	47.9	8.57	77%/86% to TrbI; plasmid R751	U67194
trbJ	49695–50468	64.2	257	29.0	9.74	75%/84% to TrbJ; plasmid R751	U67194
trbK	50478–50696	63.5	72	7.7	9.53	64%/75% to TrbK; plasmid R751	U67194
trbL	50708–52420	68.5	570	55.1	4.56	81%/86% to TrbL; plasmid R751	U67194
trbM	52438–53016	65.1	192	21.5	9.19	75%/84% to TrbM; plasmid R751	U67194
trbN	53018–53653	68.6	211	22.6	9.58	84%/89% to TrbN; plasmid R751	U67194
trbO	53683–53952	64.4	89	9.9	10.50	88%/92% to TrbO; plasmid R751	U67194
trbP	53964–54647	66.5	227	25.0	10.34	83%/92% to TrbP plasmid R751	U67194
upf30.5	54663–55106	65.1	147	15.5	7.26	68%/77% to Upf30.5; plasmid R751	U67194
upfA	55130–55411	60.3	93	9.8	6.03	–	–
upf31.0	55607–56281	66.4	224	25.4	6.19	71%/73% to Upf31.0; plasmid R751	U67194
upfB	56337–56885	57.7	182	20.6	9.69	–	–
parA[a]	57326–56854	67.0	156	17.1	9.57	79%/88% to ParA; plasmid RP4	M59825
bla $_{NPS-1}$	57504–58286	52.4	260	29.3	8.99	100%/100% to group 2d β-lactamase NPS-1; *Pseudomonas aeruginosa* plasmid pMLH50	AY027589
tniC	59008–58385	63.5	207	22.7	10.35	100%/100% to TniC; plasmid R751	U67194
traC-2	64150–59555	67.3	1531	160.0	5.33	58%/64% to TraC2; plasmid R751	U67194
traD	64552–64154	71.3	132	13.9	3.49	58%/72% to TraD; plasmid R751	U67194
traE	66634–64571	66.2	687	75.1	9.34	79%/85% to TraE; plasmid R751	U67194
traF	67185–66649	64.4	178	19.1	9.82	85%/92% to TraF; plasmid R751	U67194
traG	69086–67182	64.8	634	69.7	9.02	93%/96% to TraG; plasmid R751	U67194
traH	69795–69385	67.6	136	14.5	3.62	78%/82% to TraH; plasmid R751	U67194
traI	71341–69083	65.7	752	68.7	10.88	85%/90% to TraI; plasmid R751	U67194
orfX	71379–71338	57.1	13	1.3	8.75	46%/62% to OrfX protein; plasmid R751	U67194
traJ	71747–71376	66.4	123	13.9	8.84	83%/86% to TraJ; plasmid R751	U67194
traK	72308–72721	60.9	137	11.3	9.56	31%/46% to TraK; plasmid R751	U67194
traL	72721–73446	59.5	241	26.5	5.50	92%/97% to TraL;plasmid R751	U67194
traM	73446–73886	65.8	146	15.8	5.28	82%/90% to TraM; plasmid R751	U67194
tnpA	76807–73922	62.0	961	107.4	9.39	100%/100% to Tn*5393c* transposase; *Aeromonas salmonicida* plasmid pRAS2	AF262622
tnpR	77002–77547	60.4	181	22.3	10.38	100%/100% to Tn*5393c* resolvase; *Aeromonas salmonicida* plasmid pRAS2	AF262622
strA	77613–78416	56.2	267	29.6	4.60	100%/100% to Tn*5393c* aminoglycoside-3″-phosphotransferase StrA; *Aeromonas salmonicida* plasmidpRAS2	AF262622
strB	78416–79252	55.9	278	31.1	4.61	100%/100% to Tn*5393c* aminoglycoside-6-phosphotransferase StrB; *Aeromonas salmonicida* plasmid pRAS2	AF262622

[a]Global amino acid sequence identities and similarities were calculated with ALIGN (Myers and Miller 1988)
[b]Pseudogene, only the 3' end of the gene is present on pB4

ginosa (Cervantes et al. 1990). It was recently demonstrated that the resistance to chromate conferred by the ChrA protein in *P. aeruginosa* is based on an efflux system that extrudes chromate from the cytoplasm (Cervantes et al. 2001). Therefore, it can be assumed that the basic mechanism encoded by the putative chromate resistance determinant of Tn*5719* is similar to that of the pUM505 and pMOL28 systems, with the ChrA protein functioning as a chromate efflux pump. However, attempts to confirm the predicted biological function of the Tn*5719* resistance determinant experimentally in *Pseudomonas* sp. B13 and in *E. coli* DH5αMCR have so far failed; no resistance to chromium, cobalt or cadmium was detectable (data not shown). The plasmid-encoded chromate resistance determinants from *P. aeruginosa* and *R. metallidurans* have also been expressed in *E. coli*, but they did not confer chromate resistance on this host organism (Cervantes et al. 2001).

A global comparison between the homologous set of chromate resistance determinants also sheds new light on the genetic organization of pUM505 from *P. aeruginosa* (Fig. 3). The previously identified hypothetical coding region downstream of the *chrA* gene from pUM505 (Cervantes et al. 1990) is obviously chimeric. The deduced protein comprises 86 amino acids which showed significant similarity to the N-terminal

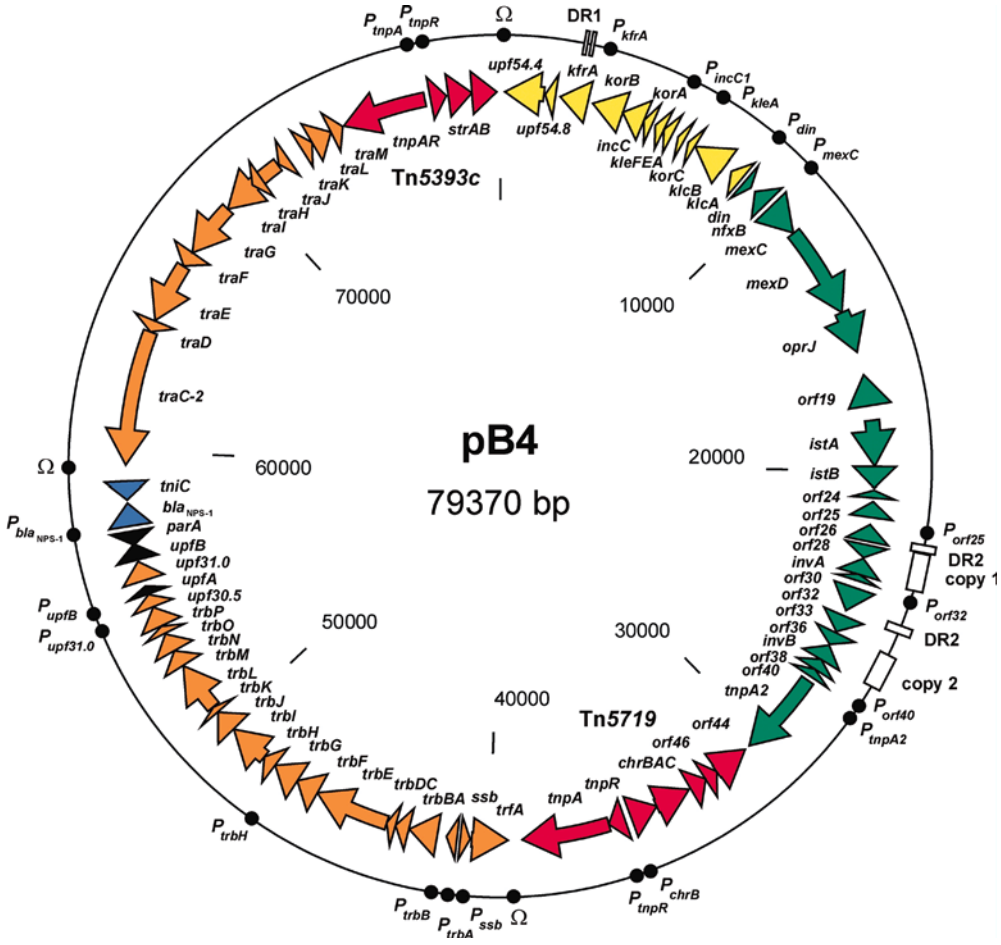

Fig. 1 Genetic map of the 79,370-bp IncP-1β resistance plasmid pB4 from a microbial community growing in activated sludge. Coding regions deduced from the complete plasmid sequence of pB4 are shown by *arrows* indicating the direction of transcription. Different colors indicate the IncP-1β backbone of pB4 (*yellow*, control and stability region; *orange*, replication, mating pair formation and DNA transfer; *black*, not present in the R751 IncP-1β backbone) and its modular 'gene load', including the putative chromate resistance transposon Tn*5719* (*red*), the streptomycin resistance transposon Tn*5393c* (*red*), the bla_{NPS-1} resistance gene region (*blue*), and a DNA segment containing the RND-MFP-OMF resistance determinant (*green*). The approximate positions of putative promoter sequences (*P*) and of rho-independent transcriptional terminators (Ω) are also marked. Long-range direct repeats (DR1 and DR2) and a 1.3-kb DNA sequence duplication (copy 1 and copy 2) are *boxed*. The lengths of the direct repeats are 63 bp (DR1) and 210 bp (DR2)

end of ChrC and the C-terminal end of the Orf44 protein from Tn*5719* (Fig. 3). This homology suggests that the intermediary gene region of pUM505 was deleted during plasmid evolution. In addition, a 21-bp remnant of the terminal inverted repeat of Tn*5719* was identified downstream of the deleted gene region (Fig. 3), indicating that the chromate resistance determinant of pUM505 was part of a Tn*5719*-like transposable element. Tn*5719* from pB4 therefore represents an ancestral transposon of the chromate resistance determinant of pUM505 from *P. aeruginosa*, and is the first chromate resistance transposon to be identified to date.

The streptomycin resistance of pB4 is encoded by the transposable element Tn*5393c*, which carries the widespread *strA* - *strB* resistance gene pair

The second mobile 'gene load' of pB4 is located in the *traM-upf54.4* intergenic region and comprises the class II Tn*3*-type transposable element Tn*5393c* (Figs. 1 and 2). This is the first description of an insertion at this location within the IncP-1β plasmid backbone. The presence of a 5-bp target site duplication, containing the native stop codon of *traM*, provided evidence that transpositional integration of Tn*5393c* had occurred at this site, which in turn resulted in the disruption of the conserved inverted repeat IR10 of the IncP-1β plasmid backbone (Table 6). The complete 5470-bp transposon region is perfectly identical at the nucleotide level to Tn*5393c* from plasmid pRAS2 of the fish pathogen *Aeromonas salmonicida* (L'Abée-Lund and Sørum 2000). Tn*5393c* encodes a transposase, a resolvase and the streptomycin resistance proteins StrA and StrB (Table 1). This genetic organization of Tn*5393c* represents the ancestral structure of a family of closely related transposons which differ mainly by the additional insertion of mobile elements (L'Abée-Lund and Sørum 2000). The linked *strA-strB* genes of Tn*5393c* encode two types of phosphotransferases, APH(3″)-Ib and APH(6)-Id, which are both required for high-level re-

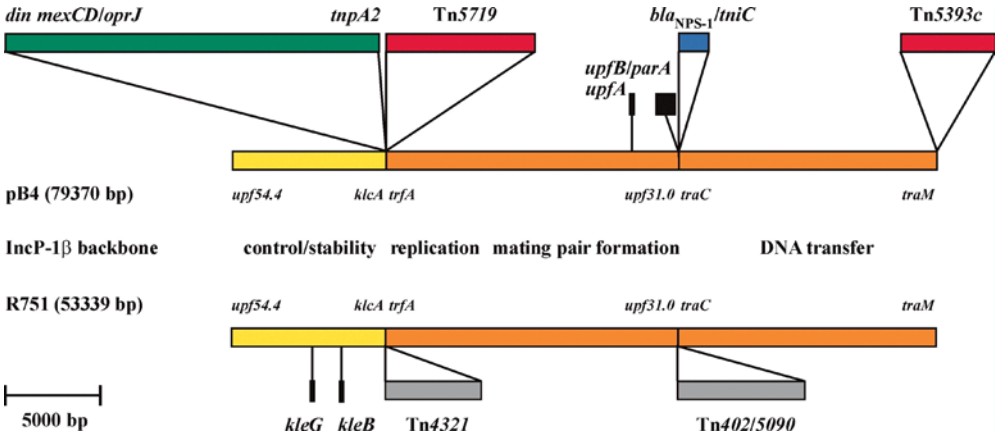

Fig. 2 Comparison of the genetic organization of the IncP-1β plasmids pB4 and R751. Linear genetic maps of pB4 (79,370 bp) and R751 (53,339 bp) are presented. Both plasmids share common sets of genes of the proposed IncP-1β backbone, which are shown by *boxes* indicating different plasmid sectors and assigned functions. Insertions within the IncP-1β backbone segments are marked by *black boxes*. The specific 'gene load' which distinguishes the two plasmid genomes is located at the junctions of the IncP-1β backbone segments. Genes flanking the plasmid sectors and the 'gene load' are shown for orientation. The color code for the different plasmid segments of pB4 is the same as that used in Fig. 1. The specific 'gene load' of R751 is shown in *grey*

sistance to the antibiotic streptomycin (Chiou and Jones 1995). A minimum inhibitory concentration (MIC) test with *Pseudomonas* sp. B13 carrying pB4 revealed increased resistance to streptomycin (MIC value greater than 40 μg/ml) relative to a susceptible plasmid-free control (5 μg/ml). These data demonstrated that the enhanced resistance was associated with the presence of pB4 in *Pseudomonas* sp. B13 (Dröge et al. 2000). The annotation of the complete pB4 nucleotide sequence therefore suggests that the identified streptomycin resistance is encoded by the *strA-strB* gene pair of Tn*5393c*.

The *strA-strB* gene pair is widely distributed in the environment and has been reported in both pathogenic and environmental bacteria from humans, animals, and plants (Sundin and Bender 1996). Most of the investigated isolates typically carried the *strA-strB* gene pair on small broad-host-range plasmids such as RSF1010, while plant-pathogenic bacteria harbored the *strA-strB* genes within variants of Tn*5393c* identified on large conjugative plasmids. The genetic association of the *strA-strB* gene pair with variants of Tn*5393c* was first demonstrated in the phytopathogenic bacteria *Erwinia amylovora*, *Pseudomonas syringae* and *Xanthomonas campestris* isolated from American agricultural habitats where streptomycin was utilized as a bactericide (Chiou and Jones 1993; Sundin and Bender 1995). Recently, several discoveries have expanded the known host range of the *strA-strB* genes associated with Tn*5393c*, since variants and remnants of the transposon have now been detected on a multi-resistance plasmid from the gram-positive human pathogen *Corynebacterium striatum* (Tauch et al. 2000) and in the chromosome of clinical isolates of *Campylobacter jejuni* and *P. aeruginosa* (Sundin 2000). The finding of the ancestral

Table 2 Putative promoter sequences identified in the pB4 sequence

Coding sequence	pB4 co-ordinates	Promoter sequence[a]	Direction[b]
kfrA	3005–3043	gtatg **ttgtag** tatgatacacgctacaa **cataca** accgc	⇐
incC1	5347–5385	aaggc **ttgacg** cgggcggagagtttagc **taaact** tctgc	⇐
kleA	6534–6573	agcgc **ttgaca** ggctagggcattttgccc **taaaat** agcac	⇐
Din	8899–8937	aagcc **ttgtcc** cttcccctggcgtgcat **tacaat** gtaat	⇒
mexC	10105–10144	caata **ttgact** catttgatgctgttgatt **gatcat** gcgcg	⇒
orf25	21631–21671	gggtt **tttaac** cgcttttataacctttatc **tataaa** cctgg	⇐
orf32	23958–23996	gcatg **atgcta** agataagactggataca **taaatt** ctagg	⇒
orf40	27396–27434	agttc **ttgaaa** tgttattctcgatccct **tagcat** cgcaa	⇐
tnpA2	27469–27507	tcacg **ttggcg** gtctggtgcagcctctc **aaaatc** cctcg	⇒
chrB	35218-35256	cttta **gttact** tctataacgttctgtag **tatata** tcaca	⇐
tnpR	35342–35380	attaa **ttgaca** tgtcccgtcaagggtca **tagatt** tcttc	⇒
Ssb	41062–41100	agttc **ttgacc** tcgaaattaggtttagc **taaact** ctagg	⇐
trbA	41079–41119	cctaa **tttcga** ggtcaagaactttagcggc **taaaat** ttcga	⇒
trbB	41717–41757	tccgt **ttgctt** gctcgtcaaaagacttcgg **tatatc** gtatg	⇒
trbH	47780–47820	cggtg **ttggtg** gaagccaagaccgcgtgac **tatctc** ccgga	⇒
upf31.0	55556–55594	cattt **atgacg** atgctggccacgtccga **tctact** ccagg	⇒
upfB	56260–56300	cgagc**tggaag** cggccgagtagctgtcaac **caaaat** ggaca	⇒
bla NPS-1	57446–57485	tacgt **ttgaat** tcactaccattgtgcgcc **gataac** aaccc	⇒
tnpA	76823–76863	ccagc **ttcata** aacaaagcgtcttgaacgc **tatcag** atttt	⇐
tnpR	76859–76898	gctgg **ttgaga** tacatttccagaggtcaa **tgcaat** cgtgg	⇒

[a]The −35 and −10 regions, which are separated by a 17- to 19-bp spacer, are indicated in *bold*

[b]The *arrows* indicate the orientation of each promoter orientation: ⇒, clockwise; ⇐, anticlockwise relative to the pB4 map shown in Fig. 1

Table 3 Conservation of putative KorA operators (O_A) between pB4 and R751

[a]The putative consensus operator sequence is shown in *bold*. Deviations from the consensus are *underlined*

Name	pB4 co-ordinates	Gene region	Operator sequence on pB4[a]	Operator sequence on R751[a]
O_A1	5349–5368	*korA*	gaga**GTTTAGCTAAAC**ttct	agaa**GTTTAGCTAAAC**catg
O_A2	3055–3074	*kfrA*	aaaa**GTT**CAGCTAAACcgct	agcg**GTTTAGCT**GAACtttt
O_A3	41064–41083	*trfA*	ttag**GTTTAGCTAAAC**tcta	gtca**GTTTAGCTAAAC**ctgc
O_A4	8870–8889	*klcA*	aaaa**GTTTAGCTAAA**Gtgct	agcaC**TTTAGCTAAA**Gctaa
O_A5	6568–6587	*kleA*	aaaa**GTTTAGCTAAA**Gcgct	agcaC**TTTAGCTAAAC**tatt

Table 4 Conservation of putative KorB operators (O_B) between pB4and R751

Name[a]	pB4 co-ordinates	Gene region	Operator sequence on pB4[b]	Operator sequence on R751[b]
O_B1	5381–5401	*korA*	catt**TTTAGCCGCTAAA**aggc	gcgt**TTTAGCGGCTAAA**aatt
O_B2	3069–3089	*kfrA*	acaa**TTTAGCCGCTAAA**aagt	tttt**TTTAGCGGCTAAA**acgc
O_B3	1506–1526	*upf54.4-upf54.8*	tagt**TTTAGCCGCTAAA**aacg	cctt**TTTAGCCGCTAAA**gcta
O_B4	71355–71375	*orfX*	gcgt**TTTAGCGGCTAAA**acgc	gctt**TTTAGCCGCTAAA**gaag
O_B5	66894–66914	*traF*	cgtc**TTTAGCGGCTAAA**acgc	cgtc**TTTAGCGGCTAAA**acgc
O_B7	53664–53684	*trbN-trbO*	ccga**TTTAGCGGCTAAA**acat	ccgaA**TTAGCGGCTAAA**catg
O_B8	49705–49725	*trbI-trbJ*	agtt**TTTAGCCGCTAAA**gtgg	agtt**TTTAGCCGCTAAA**ttgg
O_B9	41565–41585	*trbB*	actt**TTTAGCCGCTAAA**gccc	aagt**TTTAGCCGCTAAA**gccc
O_B10	41096–41116	*trfA*	gaac**TTTAGCGGCTAAA**attt	gaac**TTTAGCGGCTAAA**attt
O_B11	8884–8904	*klcA*	aggc**TTTAGCGGCTAAA**aagt	aaac**TTTAGCCGCTAAA**ctgg
O_B12	6613–6633	*korC-kleA*	aagt**TTTAGCGGCTAAA**gttt	agac**TTTAGCCGCTAAA**gcct

[a]Operators O_B6 and O_B13 are not present on pB4 and R751, but are found on the IncP-1α plasmid RP4
[b]The putative consensus operator sequence is shown in *bold*. Deviations from the consensus are *underlined*

Table 5 Conservation of putative KorC operators (O_C) between pB4 and R751

Name	pB4 co-ordinates	Gene region	Operator sequence on pB4[a]	Operator sequence on R751[a]
O_C1	8843–8865	*klcA*	gat**TAGG**GCATTTCGCCCTGgtc	tat**TAGGACAAAATGTCCTA**acc
O_C2	6540–6562	*kleA*	ggc**TAGG**GCATTTTGCCCTAaaa	tat**TAGGACAAAATGTCCTA**gcc

[a]The putative consensus operator sequence is indicated in *bold*. Deviations from the consensus of R751 are *underlined*

Table 6 Conserved inverted repeat sequences of the pB4 plasmid genome

Name[a]	pB4co-ordinates[b]	Gene region	Inverted repeat sequence on pB4[c]	Associated restriction site
IR1	39350-39375	*oriV-trfA*	CTCGAG CATCGCCGCTTCTGACGAGG	*Xho*I
IR2	39291-39316	*oriV-trfA*	CTGCAG CATCGCCATTTCCGGCGATA	*Pst*I
IR3	39253-39278	*oriV-trfA*	GTCGAC CATCGCCAGTTCCGACGATG	*Sal*I
IR4	n.a.	*oriV-trfA*	Not presenton pB4	–
IR5	39206-39232	*oriV-trfA*	GAGCTCG CATCGCCATTTCTGACGATG	*Sac*I
IR6	7176-7201	*klcB*	CTGCAG CATCGCCAGGAATGACGACG	*Pst*I
IR7	101-126	*upf54.4-traM*	CTCGAG CCTCGCCGTTTTCGACGATG	*Xho*I
IR8	52-77	*upf54.4-traM*	CTGCAG CATCGTCATTCCCGACGATG	*Pst*I
IR9	18-44	*upf54.4-traM*	TGGCCAG CCTCGCCAGTTCCGGCGATG	*Msc*I
IR10[d]	73876-73883;79359-6	*upf54.4-traM*	GTCGAC CATCGCCATTTACAGCGATG	*Sal*I
IR11	59478-59503	*traC-2*	CTCGAG CATCGCCGCATTCGACGATG	*Xho*I
IR12	59429-59454	*traC-2*	CTGCAG CATCGCCGTTTCCGACGATG	*Pst*I
IR13	n.a.	*traC-2*	Not presenton pB4	–
IR14	59390-59415	*traC-2*	GTCGAC CATCGCCAGTTCCGACGATG	*Sal*I
IR15	59343-59370	*traC-2*	GGATCTCGCATCGCCATTTCTGACGATG	(*Bam*HI/*Bgl*II) [e]
IR16	55550-55576	*upf31.0*	TGGCCAG CATCGTCATAAATGGCGATG	*Msc*I
IR17	n.a.	*upf31.0*	Not presenton pB4	–
IR18	55506-55531	*upf31.0*	CTGCAG CATCGCCAGGAACGGCGATG	*Pst*I

[a]The inverted repeats are named in accordance with the designations for R751 (Thorsted et al. 1998)
[b]n.a., not applicable
[c]The inverted repeats are located adjacent to theassociated restriction sites, which are *underlined*
[d]Inverted repeat IR10 is disrupted by the integration of Tn*5393c*
[e]The associated restriction site differs at a singleposition from the recognition sites for *Bam*HI and *Bgl*II

transposon Tn*5393c* on the IncP-1β plasmid pB4 implies that the *strA-strB* gene pair can be disseminated between bacteria in distantly related habitats using either a conjugative plasmid or a transposable element. The presence of Tn*5393c* on pB4 suggests once more that horizontal gene transfer events between human,

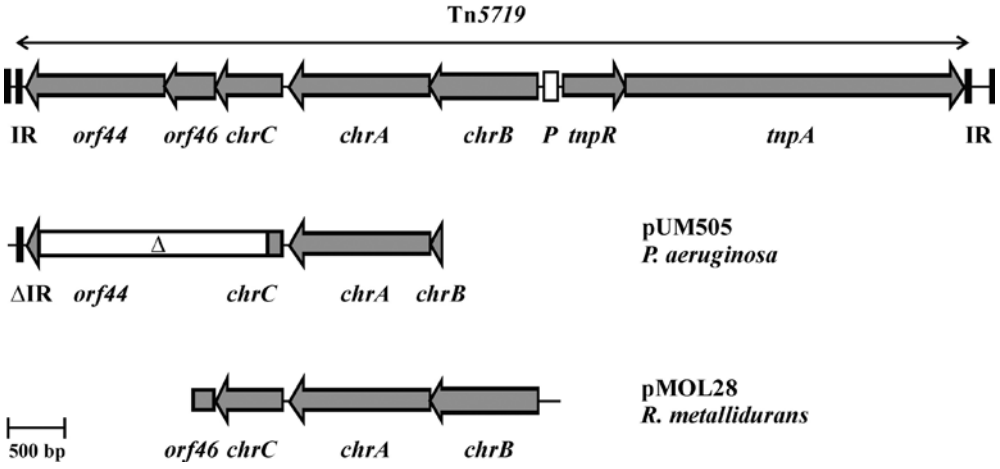

Fig. 3 Genetic organization of the putative chromate resistance determinant encoded by transposon Tn*5719* from pB4. The genetic map of Tn*5719* is compared with the chromate resistance gene regions from the *Pseudomonas aeruginosa* plasmid pUM505 and from the *Ralstonia metallidurans* plasmid pMOL28. Coding regions are shown by *arrows* indicating the direction of transcription. The predicted promoter sequence (*P*) upstream of the *chrB* gene of Tn*5719* is marked by a *box*. The conserved 38-bp terminal inverted repeats (IR) characteristic of Tn*3*-like transposons are shown by *black boxes*. Tn*5719* is flanked by a second set of inverted repeats representing another class II transposon which was designated Tn*5720*. The annotation of the pUM505 sequence from *P. aeruginosa* is based on GenBank Accession No. M29034. The translated nucleotide sequence upstream of the *chrA* gene shows 99% similarity to the C-terminal end of the ChrB protein from pB4. The hypothetical coding region downstream of *chrA* from pUM505 is obviously chimeric and consists of the 5′ end of *chrC* (amino acids 1 to 41) and the 3′ end of *orf44* (amino acid residues 42 to 86), suggesting that the intermediary gene region was deleted during plasmid evolution (Δ). In addition, a 21-bp remnant of the terminal inverted repeat of Tn*5719* (ΔIR) is located downstream of the deleted gene region on pUM505. Only the 3′end of an *orf46*-like gene was sequenced on pMOL28 from *R. metallidurans*

animal, and plant-associated bacteria have occurred, and it provides another example of the wide range of transferability of the *strA-strB* streptomycin resistance determinant.

The antibiotic resistance gene bla_{NPS-1}, which encodes a class D oxacillinase, is flanked by remnants of an integron structure

Another DNA segment encoding an antibiotic resistance determinant was found downstream of the *traC-2* gene of pB4 (Figs. 1 and 2). This DNA segment comprises the bla_{NPS-1} resistance gene and *tniC*, which is an integral part of the transposition module of the integron Tn*402/5090* from R751 (Radström et al. 1994). Interestingly, the bla_{NPS-1} gene is flanked by a 101-bp stretch of DNA with perfect sequence identity to the 5′-end of integrons, including the 25-bp terminal inverted repeat (Hall et al. 1994). The conserved integron terminus is located adjacent to a *parA* -like pseudogene (Table 1), suggesting that this gene was disrupted by an integron insertion at this site. Likewise, integron Tn*402/5090* was integrated downstream of the *traC* gene in R751 (Fig. 2; Thorsted et al. 1998; Radström et al. 1994). It is noteworthy that the bla_{NPS-1} gene does not represent an integron gene cassette, since characteristic structural features such as the 59-bp element (Recchia and Hall 1995) are obviously absent from the pB4 sequence. In addition, the bla_{NPS-1} gene is not expressed from a conserved integron promoter, but rather from its own potential promoter sequence which was identified between the integron terminal end and the bla_{NPS-1} coding region (Table 2 and Fig. 4).

BLAST database searches revealed that the nucleotide sequence of bla_{NPS-1} from pB4 is virtually identical (99.7%) to a recently sequenced resistance gene from the *P. aeruginosa* plasmid pMLH50 (Table 1). The sequence identity between the two resistance regions ends 40 bp downstream of the bla_{NPS-1} stop codon, indicating the absence of a *tniC* gene immediately downstream of bla_{NPS-1} from pMLH50 (Fig. 4). In an early study, pMLH50 was shown to encode the β-lactamase NPS-1, which was characterized only in biochemical terms (Livermore and Jones 1986). The NPS-1 protein sequence deduced from bla_{NPS-1} of pB4 and pMLH50 differs by only eight amino acids from the sequence of the β-lactamase LCR-1 encoded by the multiresistance transposon Tn*1412* on the *P. aeruginosa* plasmid pMG76 (Levesque and Jacoby 1988). The LCR-1 protein was previously classified among the oxacillin-hydrolyzing enzymes (OXA-type β-lactamases) which are frequently observed in *P. aeruginosa* isolates (Naas and Nordmann 1999). The bla_{LCR-1} resistance region of pMG76 is highly similar at the nucleotide level to the bla_{NPS-1} resistance determinant of pB4 (Fig. 4). The region of homology includes not only the coding sequences for bla_{NPS-1} and bla_{LCR-1} but also the 101-bp segment with similarity to conserved integron terminal ends and the *tniC* gene which is disrupted by a composite aminoglycoside resistance transposon in Tn*1412* (Fig. 4). This nucleotide sequence similarity within the flanking regions of bla_{NPS-1} and bla_{LCR-1} strongly indicates that both resistance determinants derived from a common mobile ancestor. Unfortunately, no larger

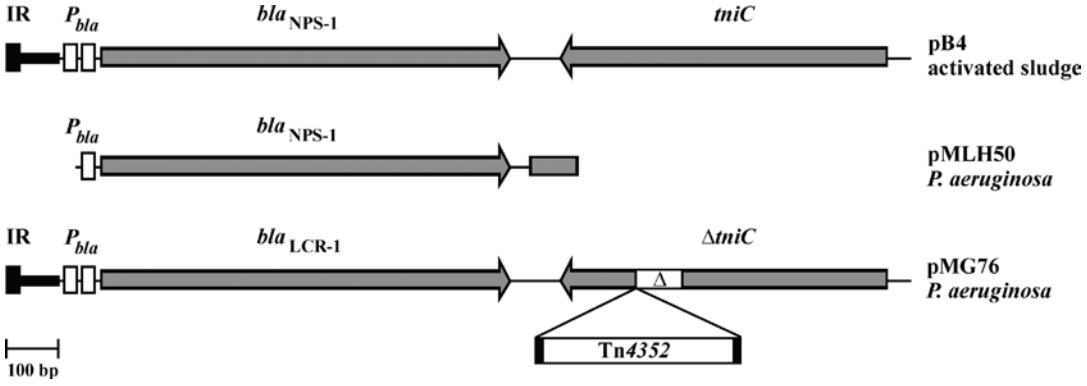

Fig. 4 Conserved genetic features of the bla_{NPS-1} and bla_{LCR-1} gene regions from pB4 and the *Pseudomonas aeruginosa* plasmids pMLH50 and pMG76. The genetic organization of the bla_{LCR-1} gene region of Tn*1412* from pMG76 was deduced from GenBank Accession No. L36547. The conserved integron fragments and the 25-bp terminal inverted repeat (IR) characteristic of integrons are marked in *black*. Promoter sequences (P_{bla}) were predicted using the Neural Network Promoter Prediction approach (Reese et al. 1996). The predicted −35 and −10 regions are marked by *boxes*. The sequenced region from pMLH50 comprises only the potential −10 region of the bla_{NPS-1} promoter. The unique downstream region of bla_{NPS-1} in pMLH50 is specifically marked by a *box*. The composite aminoglycoside resistance transposon Tn*4352* is integrated into the *tniC* gene of Tn*1412*. The *tniC* gene of Tn*1412* is further characterized by an 89-bp deletion (Δ) near the 3′ end of the coding region. Note that the 2680-bp transposon Tn*4352* is not drawn to scale

Table 7 Minimum inhibitory concentrations for *E.coli* TOP10 carrying the cloned bla_{NPS-1} gene

Antibiotic	Minimum inhibitory concentration (μg/ml) for *E.coli* TOP10 carrying the indicated plasmid	
	pZErO-2 (control)	pZErO-bla_{NPS-1}
Amoxicillin	4	> 256
Azlocillin	4	> 256
Aztreonam	≤ 0.1	0.5
Cefaclor	1	16
Cefadroxil	4	8
Cefamandole	1	4
Cefazolin	1	16
Cefotaxime	≤ 0.1	≤ 0.1
Cefoxitin	1	1
Cefuroxime	1	1
Cefoperazone	1	16
Ceftriaxone	≤ 0.1	≤ 0.1
Piperacillin	0.5	> 256

flanking sequences were determined on pMLH50 which would allow a more detailed comparison between the genetic environments of bla_{NPS-1} and bla_{LCR-1}.

The numerous β-lactamase protein sequences determined to date can be divided into four molecular classes, designated A–D, based on their sequence similarity. Structural analysis and biochemical characterization revealed that the NPS-1 protein belongs to class D (Livermore and Jones 1986; Pai and Jacoby 2001). Class D β-lactamases usually confer resistance to the antibiotic amoxicillin and their enzymatic activity is generally not inhibited by clavulanic acid (Naas and Nordmann 1999). Initial antimicrobial susceptibility tests with *Pseudomonas* sp. B13 carrying pB4 revealed that the plasmid mediates resistance to the β-lactam antibiotic amoxicillin (Dröge et al. 2000). In addition, cloning of the bla_{NPS-1} gene in *E. coli* and subsequent MIC determinations by a macrobroth dilution method provided experimental evidence that bla_{NPS-1} confers high-level resistance to amoxicillin, azlocillin, and piperacillin in *E. coli* TOP10 (Table 7). In the presence of clavulanic acid, amoxicillin showed a slightly decreased antimicrobial activity in a disk diffusion assay, indicating that it weakly inhibits the NPS-1 β-lactamase.

Interestingly, most of the currently known oxacillinase genes are located on large transferable plasmids. The resistance determinants are integral parts of transposons or they are genetically organized as gene cassettes which are located in the variable regions of integrons (Recchia and Hall 1995; Naas and Nordmann 1999). Due to their location on horizontally mobile elements, oxacillinase genes can be rapidly transferred between bacterial species. In addition, the frequent clinical use of clavulanic acid-containing β-lactam antibiotic combinations may favor the emergence of oxacillinases in human pathogens due to their weak inhibition by clavulanic acid. The acquisition and evolution of the pB4 segment carrying the bla_{NPS-1} gene region may reflect such selection for the presence of an oxacillinase resistance gene within a plasmid genome.

A tripartite antibiotic efflux system of the RND-MFP-OMF type confers erythromycin and roxithromycin resistance in *Pseudomonas* sp. B13

The most remarkable 'gene load' on pB4 is part of a 21.6-kb DNA segment located between the *klcA* gene and the putative chromate resistance transposon Tn*5719* (Fig. 1). The right end of this DNA segment is clearly characterized by the presence of a conserved 38-bp terminal inverted repeat sequence characterisitc of Tn*3*-like transposons. The putative transposon terminus is located adjacent to the 3′-end of the *tnpA2* gene encoding a Tn*3*-like transposase (Table 1). This genetic organization implies that the DNA segment might have been

transferred to pB4 by transpositional cointegrate formation with an unknown replicon, followed by genetic rearrangements. The integration site on pB4 is thus located only a few nucleotides upstream of Tn*5719* (Figs. 1 and 2). The left boundary of the DNA segment is not precisely defined but seems to lie somewhere between *klcA* and the *din* gene (Fig. 2). The integrated DNA segment mainly encodes proteins of unknown function, with the exception of a DNA-damage-inducible protein, transposase subunits, and a duplicated DNA-invertase (Table 1). Of particular interest is the presence of the genes *mexCD* and *oprJ*; the predicted products of these genes displayed 78–87% global amino acid sequence similarity to components of a multidrug efflux determinant previously identified in the *P. aeruginosa* chromosome (Table 1). The predicted resistance determinant of pB4 encodes a multidrug efflux protein of the resistance-nodulation-division (RND) family, a periplasmic membrane fusion protein (MFP), and an outer membrane factor (OMF), representing the three components of an RND-MFP-OMF multidrug resistance determinant (Zgurskaya and Nikaido 2000; Poole 2001a, 2001b). Since tripartite multidrug efflux systems of the RND-MFP-OMF type are typically encoded on the chromosomes of gram-negative bacteria (Poole 2001a), the corresponding DNA segment of pB4 might have originated from the chromosome of a hitherto unknown gram-negative microorganism.

A topology prediction for the MexD efflux protein encoded by pB4 suggested twelve hydrophobic segments, each with a minimum length of 20 amino acids, that are proposed to be transmembrane α-helices (data not shown). A further characteristic feature of the MexD protein is the presence of two large extracytoplasmic domains (each consisting of about 300 amino acids) between helices one and two and transmembrane segments seven and eight. The 1047-amino acid residues of the MexD protein are thus distributed in three subcelluar compartments: 124 residues (11.8%) are located in the cytoplasm, 650 residues (62.1%) in the periplasm, and 273 residues (26.1%) in the transmembrane segments. The cytoplasmic domain of MexD consists of five short loops and the N- and C-terminal ends. This topology prediction is consistent with the topological features of RND family proteins, which have been experimentally confirmed for the MexD efflux protein from *P. aeruginosa* (Gotoh et al. 1999). In addition, the regulatory gene *nfxB* was identified upstream of the putative resistance gene cluster in pB4 (Fig. 1 and Table 1). The NfxB protein contains an N-terminal helix-turn-helix DNA-binding motif (amino acids 24 to 45) and shows 71% global amino acid sequence similarity to the NfxB repressor protein from *P. aeruginosa* (Table 1). The *nfxB-mexC* intergenic region of pB4 contains a palindromic 15-bp inverted repeat (co-ordinates 10094 to 10123) overlapping the putative −35 promoter region of the *mexCD-oprJ* gene cluster (Table 2). This palindromic inverted repeat sequence might represent a binding site for NfxB.

Antimicrobial resistance mediated by the *mexCD-oprJ* determinant of *P. aeruginosa* is generally not detectable in wild-type cells under standard laboratory conditions, but expression of the efflux system occurs in *nfxB* mutant strains (Poole 2001b). The *nfxB* gene is located immediately upstream of the *mexCD-oprJ* genes in the *P. aeruginosa* chromosome and encodes a repressor of the *mexCD-oprJ* gene cluster (Poole et al. 1996). Expression of the antibiotic resistance determinant thus results from derepression of the efflux genes by repressor inactivation. The MexCD-OprJ antibiotic efflux system of *P. aeruginosa* confers resistance against a variety of antimicrobial agents, including macrolide antibiotics, chloramphenicol, novobiocin, tetracycline, trimethoprim, and some β-lactams such as cefoperazone, ceftazidime, and fourth-generation cephems (Poole 2001b). Furthermore, the MexCD-OprJ system of *P. aeruginosa* has been shown to be an inducible resistance determinant; inducers include tetraphenylphosphonium chloride, ethidium bromide, rhodamine 6G, and acriflavine (Morita et al. 2001). Interestingly, erythromycin, chloramphenicol, tetracycline, and fluoroquinolones did not act as inducers, although they were recognized as substrates by the efflux system. The known RND-MFP-OMF determinants from *P. aeruginosa* thus pump out a variety of substrates, but they differ significantly in their specificities (Poole 2001b). Therefore, it was of great importance to determine the substrate spectrum of the *mexCD-oprJ* determinant present on plasmid pB4.

Initial antimicrobial susceptibility assays with *Pseudomonas* sp. B13 carrying pB4 revealed that the plasmid encodes streptomycin, amoxicillin and erythromyicn resistances (Dröge et al. 2000). The presence of the *strA-strB* streptomycin resistance gene pair and the $bla_{\text{NPS-1}}$ oxacillinase gene suggests that the *mexCD-oprJ* determinant of pB4 might be responsible for the observed resistance to erythromycin in *Pseudomonas* sp. B13. To provide experimental evidence for this conclusion, the *nfxB-mexCD-oprJ* determinant of pB4 and an *nfxB* deletion derivative were cloned in *E. coli* in the broad-host-range vector pJP2. The *nfxB* deletion derivative was constructed to ensure constitutive derepression of the *mexCD-oprJ* genes during susceptibility testing. The resulting plasmids pJP2-*mex* and pJP2-Δ*nfxB* were subsequently transferred to *Pseudomonas* sp. B13, *S. meliloti* FP2, and *E. coli* S17-1. Disk diffusion assays with a set of antimicrobial agents confirmed that the complete resistance determinant and the *nfxB* deletion derivative both conferred resistance against the macrolide antibiotic erythromycin in *Pseudomonas* sp. B13, but not in *S. meliloti* FP2 or in *E. coli* S17-1. To further characterize the substrate spectrum of the cloned resistance gene regions on pJP2, a macrobroth dilution assay using macrolide-lincosamide antibiotics was performed with the plasmid-bearing derivatives of *Pseudomonas* sp. B13 (Table 8). The deduced MICs of the macrolide-lincosamide antibiotics indicated that the cloned efflux system not only conferred erythromycin but also roxithromycin resistance on *Pseudomonas* sp. B13. The *mexCD-oprJ* determinant

Table 8 Minimum inhibitory concentrations of macrolide-lincosamide antibiotics for *Pseudomonas* sp. B13

Antibiotic	Minimum inhibitory concentration (μg/ml) for *Pseudomonas* sp. B13 carrying the indicated plasmid		
	pJP2 (control)	pJP2-*mex* (*nfxB-mexCD-oprJ*)	pJP2-Δ*nfxB* (*mexCD-oprJ*)
Clindamycin	256	256	256
Erythomycin	4	128	64
Josamycin	128	128	128
Lincomycin	256	256	256
Roxithromycin	1	128	16
Tylosin	128	128	128

of pB4 does not confer resistance to other structurally related antibiotics in *Pseudomonas* sp. B13. This may result either from its distinctive substrate spectrum or be due the already high intrinsic resistance of the host strain to macrolides and lincosamides (Table 8). Likewise, the intrinsic resistance of *S. meliloti* FP2 and *E. coli* S17-1 to macrolide-lincosamide antibiotics is very high (MIC greater than 128 μg/ml), which might explain the failure to detect an erythromycin and roxithromycin resistance phenotype in these species. In addition, no induction of the *mexCD-oprJ* determinant by low concentrations of acriflavine, rhodamine 6G or ethidium bromide was observed in *Pseudomonas* sp. B13; no changes in the minimum inhibitory concentrations of a set of antimicrobials were detectable under these conditions (data not shown). In conclusion, only a narrow substrate spectrum, encompassing the macrolide antibiotics erythromycin and roxithromycin, was identified for the predicted RND-MFP-OMF antibiotic efflux system of pB4 and these resistances were only detectable in *Pseudomonas* sp. B13.

Nowadays, the wealth of genome sequence information present in public databases allows one to predict a number of possible homologs of multidrug resistance determinants, indicating that RND-MFP-OMF efflux systems are widespread in the chromosomes of gram-negative bacteria. Although their contribution to intrinsic or acquired resistance has not been confirmed experimentally, many of the predicted determinants will not play a significant role in antibiotic resistance (Poole 2001a). The functional analyses presented in this study provide evidence that the RND-MFP-OMF determinant identified on the IncP-1β plasmid pB4 obviously contributes to an acquired antibiotic resistance phenotype directed against the clinically relevant antibiotics erythromycin and roxithromycin in *Pseudomonas* sp. B13.

Concluding remarks

Nucleotide sequence analysis and computer-assisted annotation of the multiresistance plasmid pB4 has revealed a novel example of the modular evolution of plasmid genomes. The overall organization of pB4 is characterized by an IncP-1β plasmid backbone and a modular 'gene load' which appears to be involved in resistance functions. The specific 'gene load' of pB4 includes the putative chromate resistance transposon Tn*5719*, the streptomycin resistance transposon Tn*5393c*, the oxacillinase gene $bla_{\text{NPS-1}}$, and a tripartite antibiotic efflux system of the RND-MFP-OMF type which conferred erythromycin and roxithromycin resistance in *Pseudomonas* sp. B13. Plasmid pB4 was initially obtained by an exogenous plasmid isolation method from an uncultured bacterium from activated sludge. Although the original host of pB4 is currently unknown, the genetic determinants on pB4 undoubtedly play an important role in acquired resistance of its potential host organisms. The important contribution of pB4 to acquired antimicrobial resistance is reflected by a set of resistance determinants which revealed different substrate spectra. Interestingly, all antimicrobial resistance genes identified on pB4 showed the highest similarity to resistance determinants from clinical isolates of *P. aeruginosa*. This similarity at the nucleotide and protein levels demonstrates that the 'gene load' of pB4 is obviously relevant for the development of multiresistance in human pathogens. In particular, the complementary substrate spectra of the $bla_{\text{NPS-1}}$ gene, the *strA-strB* gene pair, and the RND-MFP-OMF efflux system – all encoded by a single plasmid genome – could eventually result in a multiresistant human pathogen upon horizontal gene transfer of pB4 by means of its own IncP-1β conjugation apparatus. In conclusion, the pB4 genome sequence analysis demonstrates that reservoirs of antimicrobial resistance genes exist in nature with hitherto unknown sets of antibiotic resistance determinants capable of conferring multidrug resistance on human pathogens.

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