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Cloning and characterization of a region responsible for the maintenance of megaplasmid pTAV3 of *Paracoccus versutus* UW1

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Abstract

Using cointegrate formation, we constructed a basic replicon of the megaplasmid/mini-chromosome pTAV3 of *Paracoccus versutus* UW1. It is composed of two adjacent modules, responsible for plasmid replication (*rep*) and partitioning (*par*). Functional analysis of the *par* region identified a determinant of incompatibility (*inc2*), whose presence is crucial for proper partitioning (the partitioning site). Database searches revealed that the only known replicon with significant homology to that of pTAV3 is encoded by the chromosome cII of *Rhodobacter sphaeroides* 2.4.1. Incompatibility studies showed that closely related basic replicons are also encoded by megaplasmids (above 400 kb) harbored by four strains of *P. pantotrophus*. Basic replicons of the pTAV3-type are able to maintain large bacterial genomes, therefore they appear to be good candidates for the construction of vectors specific for Alphaproteobacteria.

Keywords: *Paracoccus versutus*; pTAV3; Megaplasmid; Partitioning site; Incompatibility

1. Introduction

Over the last decade much attention has been given to the identification and analysis of mechanisms that ensure stable maintenance of various replicons in bacterial cells. Bacterial plasmids, which are dispensable for growth of their hosts, have been the most convenient models for such studies. It was shown that plasmids encode at least three different kinds of stabilizing mechanisms. These are (i) active partitioning systems (*par*) involved in specific positioning of plasmid molecules to certain defined subcellular locations and their

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efficient segregation to daughter cells at cell division, (ii) multimer resolution systems that increase copy number of plasmid monomers, and thus optimize their random segregation, and (iii) addiction systems responsible for post-segregational elimination of plasmid-less cells from a bacterial population (reviewed by Zielenkiewicz and Ceglowski, 2001).

Much less is known about the maintenance systems specific for larger replicons—megaplasmids and chromosomes. This process is especially important for representatives of Alphaproteobacteria, many of which contain more than one chromosome (Jumas-Bilak et al., 1998). The first bacterial species shown to possess a complex genome, consisting of two circular chromosomes [termed cI (3188 kb) and cII (943 kb)] and five plasmids (ranging in size from 42 to 113 kb), was *Rhodobacter sphaeroides* 2.4.1 (Choudhary et al., 1997 and http://mmg.uth.tmc.edu/sphaeroides/). Other examples of bacteria with complex genomes are the related *Paracoccus denitrificans* and *P. pantotrophus*, which carry 3–4 large replicons designated CI–CIV, in the size range 0.5–2.2 Mb (Winterstein and Ludwig, 1998).

So far, the only characterized replicons specific for mega-sized plasmids (and some linear chromosomes) of Alphaproteobacteria belong to the so-called *repABC* family (e.g., Bartosik et al., 1998; Ramírez-Romero et al., 1997; Turner and Young, 1995). Within the replicons of this type, the genes coding for active partitioning proteins (*repA* and *repB*) and the structural gene for a replicator protein (*repC*) are clustered in an operon. This ensures a unique structural and regulatory relationship between genes involved in these processes (Bartosik et al., 2001; Ramírez-Romero et al., 2001).

A few years ago we initiated studies on the mobile elements of *Paracoccus versutus* (strain UW1), a bacterium which is very closely related to *R. sphaeroides*. *P. versutus* also has a multi-replicon genomic organization, since in addition to the chromosome(s), it carries three replicons: plasmid pTAV1 (107 kb) and two megaplasmids pTAV3 and pTAV4 (>400 kb), which can be visualized by DNA electrophoresis following in-gel lysis.

Detailed analysis of pTAV1 revealed the composite nature of this plasmid, since it carries two replicator regions, one of which belongs to the aforementioned *repABC* family (Bartosik et al., 1998, 2001). We also analyzed the replicator region of megaplasmid pTAV3, cloned in the form of an unstable mini-replicon pTAV400 (5.6 kb) (Bartosik et al., 2002a). This analysis led to the identification of the gene *rep* coding for the replication initiator protein and the origin of replication (*oriV*), located upstream of the *rep* gene. In the opposite transcriptional orientation to the *rep* gene, we identified an incomplete open reading frame, coding for part of a putative ParA partitioning ATPase. The intergenic region between *rep* and the putative *parA* appeared to contain a strong determinant of incompatibility (*inc1*), whose introduction into *P. versutus* UW1 resulted in displacement of the parental pTAV3. In addition, we found that strains deprived of pTAV3 lack some undefined housekeeping genes required for growth in minimal media. This megaplasmid can therefore be referred to as a mini-chromosome (Bartosik et al., 2002a).

Here we present the results of an experimental approach aimed at isolation and characterization of the basic replicon of pTAV3, with special focus on the partitioning system. Our analyses resulted in the identification of a novel class of pTAV3-like basic replicons, able to maintain large bacterial genomes, which appear to be good candidates for the construction of specific vectors for this group of bacteria (e.g., for the creation of genomic libraries).

2. Materials and methods

2.1. Bacterial strains, plasmids, and culture conditions

Bacterial strains used in this study were: *P. versutus* UW1R (Rif^r derivative of the wild-type UW1; carries pTAV1, pTAV3, and pTAV4; Bartosik et al., 2001), UW400 (pTAV3-less derivative of UW1; Bartosik et al., 2002a), *P. pantotrophus* DSM 11073R (Bartosik et al., 2002b), DSM 11072R, DSM 65R, and LMD 82.5R (Bartosik et al., 2002c) as well as *Escherichia coli* strains: TG1 (used for cloning experiments; Sambrook and Russell, 2001), DH5α (host strain for pRK2013; Ditta et al., 1980), and M15 (Qiagen).

Bacteria were grown in Luria–Bertani (LB) medium (Sambrook and Russell, 2001) at 30 °C (*Paracoccus* spp.) or 37 °C (*E. coli*). The minimal medium for the propagation of *Paracoccus* spp. was prepared as described by Wood and Kelly (1977). When necessary, the medium was supplemented with antibiotics: ampicillin (Ap), 100 μg/ml; spectinomycin (Sp), 50 μg/ml; kanamycin (Km), 50 μg/ml; rifampicin (Rif), 50 μg/ml; tetracycline (Tc), 3 μg/ml for *P. versutus*, and 20 μg/ml for *E. coli*. The plasmids used in this study are listed in Table 1.

Table 1
Plasmids used in this study

Plasmid	Relevant characteristics	References or source
pTAV3	Megaplasmid/mini-chromosome (above 400 kb) of *P. versutus* UW1	Bartosik et al. (2002a)
pTAV400	Km^r; mini-replicon carrying 4.3 kb *Eco*RI fragment of pTAV3 and Km^r cassette (1.3 kb)	Bartosik et al. (2002a)
pABW1	Km^r; mobilizable cloning vector, ColE1 origin of replication, *oriT* of RK2	Bartosik et al. (1997)
pABW421	Km^r; pABW1 derivative carrying 1.3 kb *Pst*I fragment of pTAV400	This study
pTAV423	Km^r; pABW1 derivative carrying 11 kb fragment of pTAV3 (containing basic replicon of pTAV3)	This study
pDBV54	Km^r; pABW1 derivative carrying 6.8 kb *Bam*HI fragment of pTAV423 (basic replicon of pTAV3)	This study
pBGS18	Km^r; cloning vector, ColE1 origin of replication	Spratt et al. (1986)
pBGS99	Km^r; pBGS18 derivative carrying 2.7 kb *Bam*HI–*Eco*RI fragment containing the *par* region of pTAV3	This study
pRK415	Tc^r; mobilizable broad-host-range cloning vector, *oriT* and origin of replication of RK2	Keen et al. (1988)
pRK415inc2	Tc^r; pRK415 derivative carrying 1.4 kb *Bam*HI–*Apa*I fragment of pTAV3 containing *inc2*	This study
pJAK327	Tc^r; pRK415 derivative carrying PCR-amplified A module of the sequence placed downstream of the *parB* gene of pTAV3	This study
pJAK196	Tc^r; pRK415 derivative carrying PCR-amplified B module	This study
pJAK574	Tc^r; pRK415 derivative carrying PCR-amplified C module	This study
pJAK503	Tc^r; pRK415 derivative carrying PCR-amplified A and B modules	This study
pJAK283	Tc^r; pRK415 derivative carrying PCR-amplified B and C modules	This study
pJAK2345	Tc^r; pRK415 derivative carrying PCR-amplified A, B, and C modules	This study
pGEM-T Easy	Ap^r; vector enabling cloning of PCR products	Promega
pGB2	Sp^r; cloning vector; pSC101 origin of replication, silencing reporter plasmid	Churchward et al. (1984)
pPDB11	Sp^r; pGB2 derivative carrying *inc2* of pTAV3 (module C)	This study
pQE30	Ap^r; plasmid for construction and expression of 6× His-tagged fusion proteins; ColE1 origin of replication	Qiagen
pPDB30/ParB	Ap^r; pQE30 derivative containing PCR-amplified *parB* gene of plasmid pTAV3	This study
pABW3	Km^r; mobilizable *E. coli–P. versutus* shuttle vector composed of pABW1 and unstable pTAV202	Bartosik et al. (2001)
pABW3/par	Km^r, pABW3 derivative carrying 4.1 kb *Bam*HI–*Kpn*I fragment with partitioning system of pTAV3	This study
pCM66	Km^r; mobilizable cloning vector carrying ColE1 and RK2 replicator regions, *oriT* of RK2	Marx and Lidstrom (2002)
pDBV51	Km^r, pCM66 derivative carrying *inc1*	This study
pDBV56	Km^r, pCM66 derivative carrying *inc2* (module C)	This study
pRK2013	Km^r, helper plasmid carrying RK2 *tra* genes	Ditta et al. (1980)

2.2. DNA manipulations

Plasmid isolation, digestion with restriction enzymes, ligation and agarose gel electrophoresis were conducted according to standard procedures (Sambrook and Russell, 2001). All enzymes were purchased from Fermentas. Megaplasmid visualization was achieved by in-gel lysis and DNA electrophoresis according to Wheatcroft et al. (1990). For Southern hybridization (Sambrook and Russell, 2001) DNA probes were labeled with digoxigenin (Roche). Hybridization and visualization of hybridization products was carried out as recommended by the supplier.

2.3. Construction of a plasmid for overproduction of a ParB(His)$_6$ fusion protein

For the construction of a plasmid expressing recombinant ParB protein, PCR was employed to amplify the coding region of *parB*. The resulting PCR product was digested with *Bam*HI and cloned in frame in the corresponding site of plasmid pQE30, generating a fusion between the *parB* gene and a vector sequence coding for six histidine residues (6× His-tag). The His-tag was placed at the *N*-terminus of ParB. Overproduction of the recombinant ParB(His)$_6$ protein in *E. coli* M15 was verified by SDS–PAGE and Western blotting with anti-His tag antibody (Qiagen). The recombinant ParB(His)$_6$ protein was purified as previously described (Bartosik et al., 2001).

2.4. DNA sequencing and sequence analysis

To determine the complete nucleotide sequence of the *par* region of pTAV3, the 2.7 kb *Bam*HI–*Eco*RI fragment of pDBV54 was cloned into vector pBGS18 to produce plasmid pBGS99 and sequenced on both strands using an automated ABI 377 DNA sequencer (Perkin–Elmer). Sequence analysis was performed with programs included in the UWGCG Package Version 8.1 (Devereux et al., 1984). Similarity searches were performed with the BLAST program (Altschul et al., 1990) provided by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST).

2.5. Introduction of plasmid DNA into bacterial cells

Competent cells for transformation of *E. coli* TG1 were prepared and transformed as described by Kushner (1978). For triparental mating three overnight cultures (cells harvested by centrifugation and washed to remove antibiotics) of (i) the donor strain *E. coli* TG1 (carrying a mobilizable plasmid), (ii) the *P. versutus* or *P. pantotrophus* strain as the recipient, and (iii) *E. coli* DH5α carrying the helper plasmid pRK2013, were mixed at a ratio of 1:2:1. An aliquot of 100 μl of this mixture was spread on a plate of LB medium. After overnight incubation at 30 °C the bacteria were washed off the plate and dilutions of the cell suspension were plated on selective LB medium containing rifampicin (selective marker of the recipient strain) and kanamycin or tetracycline to select for transconjugants. Spontaneous resistance of the recipient strains to Km and Tc was not detected.

2.6. PCR amplification

For amplification of the regions termed A, B, and C, carrying a potential partitioning site, the following pairs of forward and reverse oligonucleotide primers were used: AL (5′-CGCAAGGA AGACTGAGGAAG-3′) and AR (5′-GGCTT ACCCGGAACAGATAC-3′), BL (5′-CGTCGCT ATGCTGGAACGCT-3′) and BR (5′-CCGCAC ATTGGACTGGCTCA-3′), and CL (5′-CGTC GCTATGCTGGAACGCT-3′) and CR (5′-CC GCACATTGGACTGGCTCA-3′). The *parB* gene of pTAV3 was amplified with primers PARBL (5′-AAGGATCCATGCCGTCAACGACGAGATC-3′) and PARBR (5′-AAGGATCCCAGTCTTC CTTGCGCTTCCA-3′). The introduced *Bam*HI restriction sites are underlined. Amplification was performed in a Mastercycler (Eppendorf) using the above primers, *OptiTaq* polymerase from Eurex (with supplied buffer) and template DNA (pDBV54). Reaction mixtures (50 μl) contained 10 ng template DNA, 2 mM $MgCl_2$, 200 μM dNTPs, 50 pmol of each primer and 0.5 U *OptiTaq* polymerase. The amplification cycle was: 96 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 53–57 °C (depending on the primers used) for

30 s and 72 °C for 1 min; the last cycle was followed by an additional annealing step and a final extension step of 10 min. PCR products were separated by 1 or 2% agarose gel electrophoresis, extracted from the gel using a DNA Gel-Out kit (DNA Gdansk) and cloned into the pGEM-T Easy vector (Promega).

2.7. DNA mobility shift assay

DNA binding reaction was performed at 37 °C for 5 min by incubating a DNA fragment (200 ng) (PCR amplified modules A, B, C, A + B, and B + C; described under Section 3) with purified RepB(His)$_6$ (700 ng) and nonspecific competitor DNA (calf thymus DNA) in binding buffer (derived from DIG Gel-shift kit; Roche). The final volume of the reaction mixture was 20 μl. The samples were loaded and separated on 2% agarose gel in Tris–borate–EDTA buffer (pH 8.3). The gels were transferred to BioBond-Plus Nylon membrane (Sigma) and the transferred DNA was detected by hybridization with a DIG-labeled PCR-amplified DNA fragment containing A + B + C modules.

2.8. Incompatibility testing

The incompatibility characteristics of two plasmids were examined by conjugal transfer (triparental mating) of the tested recombinant Tc^r plasmids based on vector pRK415 (containing PCR-amplified or restriction fragments of pTAV3) into the recipient *P. versutus* strain UW1R (Rif^r derivative of the wild-type strain UW1). The plasmid pattern of transconjugants was verified by screening 10 colonies of each strain using a rapid alkaline extraction procedure with agarose gel electrophoresis as well as by in-gel lysis and electrophoresis according to Wheatcroft et al. (1990).

2.9. Plasmid stability

The stability of plasmids during growth in non-selective conditions was tested as described previously (Bartosik et al., 2002a). Briefly, stationary phase cultures were diluted in fresh medium without antibiotic selection and cultivated for approximately 30 generations. Samples were diluted and plated onto solid medium in the absence of selective antibiotics. Two hundred colonies were tested for the presence of the Km^r or Tc^r markers by replica plating. The retention of plasmids, after approximately 30 generations, was determined from the percentage of kanamycin- or tetracycline-resistant colonies.

2.10. Nucleotide sequence accession number

The complete annotated nucleotide sequence of the basic replicon of megaplasmid pTAV3 was deposited at GenBank with Accession no. AF390867.

3. Results and discussion

3.1. Cloning of a basic replicon of pTAV3

The replicator region of the mini-chromosome pTAV3 was previously cloned within a 4.3 kb *Eco*RI restriction fragment (mini-replicon pTAV400; Fig. 1). To characterize the basic replicon of this mini-chromosome, carrying all information necessary for its stable maintenance, we decided to clone a larger restriction fragment of pTAV3, containing both the *rep* and *par* regions. Because of difficulties experienced in isolating such a large replicon (>400 kb), the construction was performed following an initial, cointegrate formation step. The cloning strategy is shown in Fig. 1.

Briefly, a 1.3 kb *Pst*I restriction fragment of pTAV3, located downstream of the *rep* gene of mini-replicon pTAV400, was cloned (in *E. coli*) into the multiple cloning site (MCS) of the mobilizable vector pABW1 (Km^r; unable to replicate in *Paracoccus* spp.). The derived plasmid (pABW421; Fig. 1) was introduced (as a suicide replicon) by triparental mating into *P. versutus* UW1R. As expected, several Km^r Rif^r transconjugants were obtained, in which (as confirmed by DNA hybridization analysis) pABW421 was integrated into pTAV3 (data not shown). Total DNA isolated from a randomly selected transconjugant, was digested with *Hin*dIII (unique restriction site present in the MCS of pABW421), and following ligation it was used to transform *E. coli* TG1. The resulting

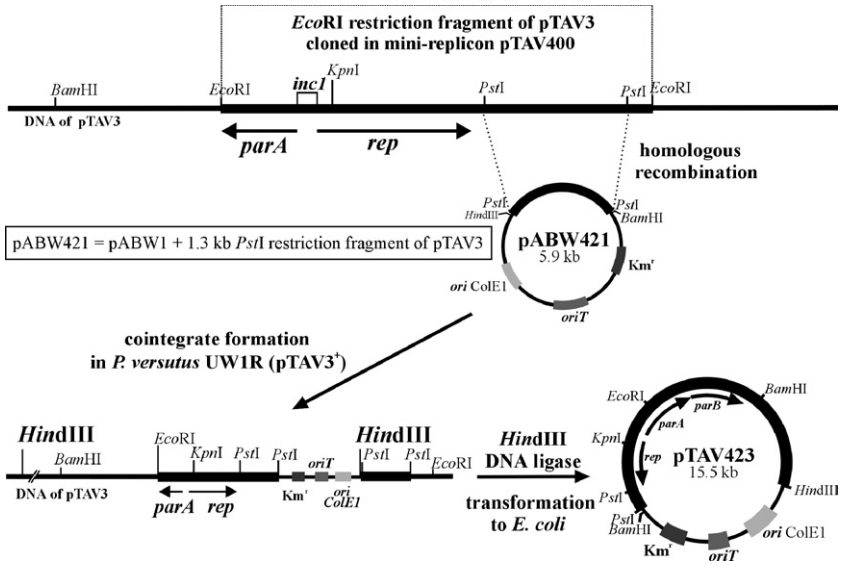

Fig. 1. Strategy for cloning a basic replicon of pTAV3. See text for details.

plasmid pTAV423 consisted of the vector pABW1 carrying an 11 kb fragment of pTAV3 (Fig. 1). This shuttle plasmid could be transferred to and stably maintained in *P. versutus* UW400 (pTAV1$^-$, pTAV3$^-$); no plasmid-less cells were detected after approximately 30 generations of growth in non-selective conditions.

In the next step the functional basic replicon of pTAV3 was localized to a 6.8 kb *Bam*HI restriction fragment of pTAV423 (Fig. 1). Introduction of this fragment into pABW1 produced the shuttle vector pDBV54 (stably maintained in *P. versutus* UW400), which was subjected to more detailed analysis.

3.2. DNA sequencing and sequence analysis

The nucleotide sequence of a 2.7 kb fragment *Bam*HI–*Eco*RI of pDBV54 was determined and compiled with the known sequence of pTAV400 (Bartosik et al., 2002a). This revealed the complete genetic information comprising the basic replicon of pTAV3 (7082 bp) (GenBank Accession No. AF390867).

In general, *par* systems consist of three elements, each of which is crucial for proper segregation. These are, two genes organized in an operon located adjacent to an essential *cis* partitioning site (reviewed by Bignell and Thomas, 2001). Analysis of the pTAV3 sequence data led to the identification, downstream of the *rep* gene, of two partially overlapping (8 bp overlap) open reading frames (ORF1 and ORF2) transcribed in the same direction (Fig. 2). Both ORF1 and ORF2 are preceded by putative ribosome binding sites (RBSs) located upstream of the respective initiation codons (ATG) within purine-rich regions (5′-AGAA GGA-3′ and 5′-GAAGGAG-3′, respectively). ORF1 (nucleotides 2274–3740) encodes a 488 aa polypeptide with a predicted molecular mass of 54.3 kDa and a p*I* value of 5.28. The overlapping ORF2 (nucleotides 1169–2281) encodes a 370 aa polypeptide with a predicted molecular mass of 41.3 kDa and p*I* value of 5.72. A comparison of the hypothetical ORF1 and ORF2-encoded products with the databases revealed their homology to a number of proteins involved in active partitioning of bacterial plasmids and their chromosomally encoded counterparts. ORF1, as previously suggested, encodes a protein with significant similarity to the SopA/ParA family of type Ia partition ATPases, while ORF2 is similar to the corresponding partitioning-site-binding proteins (Bignell and Thomas, 2001). The highest similarity levels were with the putative partition proteins of (i) the cII chromosome of *R. sphaeroides* 2.4.1

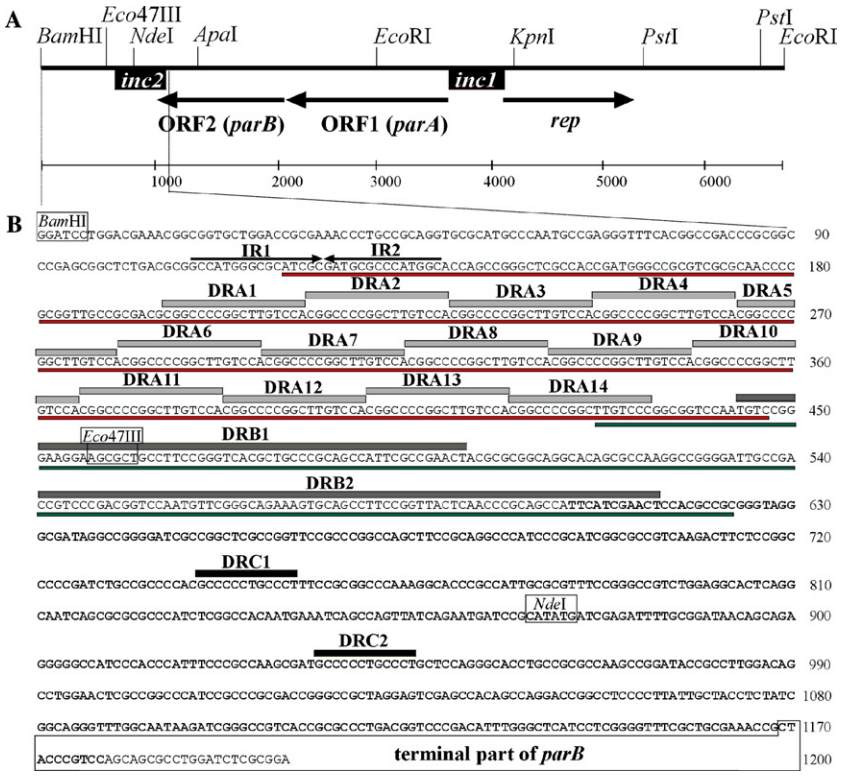

Fig. 2. (A) Genetic organization of a basic replicon of pTAV3. The arrows indicate the *par* and *rep* genes and their transcriptional orientation. The location of the *inc1* and *inc2* regions and the restriction sites used for plasmid construction are indicated. (B) Nucleotide sequence of the region downstream of the *par* operon of pTAV3 containing a partitioning site. The inverted repeat sequences (IR1 and IR2) are indicated with arrows. The sets of repeat sequences (DRA1-14, DRB1-2, and DRC1-2) are marked by shaded boxes. The nucleotide sequence containing *inc2* (module C) is bolded, while the sequence of modules A and B are underlined with red and green thick lines, respectively. Relevant restriction sites are indicated. The numbers to the right refer to the nucleotide sequence of the basic replicon of pTAV3 (GenBank Accession No. AF390867).

(Rsph023117 and Rsph023116; GenBank Accession Nos. ZP_00007135 and ZP_00007134, respectively, and http://mmg.uth.tmc.edu/sphaeroides/) (86 and 63% similarity, respectively), (ii) plasmid pRS241a (113 kb) of *R. sphaeroides* 2.4.1 (Rsp h023731 and Rsph023730; GenBank Accession Nos. ZP_00207036 and ZP_00207035, respectively, and http://mmg.uth.tmc.edu/sphaeroides/) (75 and 51% similarity, respectively), and (iii) plasmid pSD20 (58 kb) of *Ruegeria* sp. (RB101 and RB102; GenBank Accession Nos. AAN05122 and AAN05123, respectively; Zhong et al., 2003) (74 and 46% similarity, respectively). The similarities of ParA and ParB of pTAV3 to their closest homologues extend over the entire length of the proteins. Some similarities were also observed with RepA and RepB partitioning proteins of the previously mentioned *repABC* type replicons [e.g., Y4cK and Y4cJ of plasmid pNGR234a of *Rhizobium* sp. NGR234 (GenBank Accession Nos. NP443803 and NP443802, respectively) (45 and 39% similarity, respectively); RepA and RepB of *Brucella suis* (GenBank Accession Nos. NP700354 and NP700355, respectively) (42 and 39% similarity, respectively).

The maintenance regions of pTAV3, chromosome cII, pRS241a and pSD20 have similar genetic organization (*rep* gene in the opposite orientation to the *par* operon); however, only the putative replication protein of chromosome cII is homologous to the Rep of pTAV3 (72% similarity and 62% identity).

To confirm the role of ORF1 and ORF2 of pTAV3 in plasmid partitioning, mutational analysis was performed. This revealed that mutations introduced within ORF1 (4 bp insertion at the *Eco*RI site; Fig. 2A) or within ORF2 (4 bp insertion at the *Apa*I site; Fig. 2A) completely abolished the ability of the analyzed system to stabilize pABW3. Based on these analyses, ORF1 and ORF2 were designated *parA* and *parB*, respectively.

The partitioning sites of many plasmid-encoded *par* systems (located downstream or upstream of the *par* operon), although different in structure, possess some characteristic features which permit their identification. Most contain iterated sequences which constitute the sites of binding for ParB-like proteins (Gerdes et al., 2000). Although the intergenic region, between the *rep* gene and *par* operon, contains the determinant of incompatibility, *inc1* (Fig. 2A), the nucleotide sequence downstream of the *parB* gene, possesses some structural features similar to other defined partitioning sites (Fig. 2B). Its analysis revealed the presence of three sets of different, directly repeated sequences. Three sequence modules (designated A, B, and C) were distinguished carrying, respectively: (i) 14 identical, tandemly repeated 17-bp sequences (5′-CGGCCCCGGCTTGTCCA-3′; DRA1-14; positions 196–433), followed by a 30-bp-long palindromic sequence, composed of two identical inverted repeats (IR1–2), (ii) two long (72 bp), 82% identical repeated sequences (DRB1-2), separated by a 39-bp spacer sequence (positions 444–501 and 541–614, respectively), and (iii) two 12-bp-long repeats (5′-GCCCCCT GCCCT-3′) (termed DRC1-2), adjacent to the *parB* gene and separated from one another by 182 bp, (positions 740–751 and 934–945, respectively) (Fig. 2B). Database searches (BLASTN) failed to reveal the presence of homologous repeat sequences within available microbial genomes.

3.3. Identification of the second determinant of incompatibility of pTAV3

To test whether the sequence placed downstream of *parB* plays an important role in plasmid maintenance, we studied its incompatibility behavior towards parental pTAV3. In general two incompatible plasmids (i.e., carrying related replication or partitioning systems) cannot be stably maintained in a bacterial cell in the absence of selective pressure. Therefore a 1.4 kb *Bam*HI–*Apa*I restriction fragment of pTAV3 (containing the 3′ end of *parB* and the region downstream; Fig. 2) was cloned into the broad-host-range vector pRK415 (Tc^r; compatible with pTAV3) in *E. coli* TG1 and the resulting construct pRK415inc2, was transferred by triparental mating into the wild-type strain *P. versutus* UW1R ($pTAV3^+$). All Tc^r transconjugants containing pRK415inc no longer carried pTAV3, which confirmed the presence of a strong *inc* region within the tested DNA fragment.

For precise localization of the *inc* region, the three previously distinguished modules (A, B, and C), as well as larger DNA fragments carrying different combinations (A + B, B + C, and A + B + C) were amplified by PCR, cloned in pRK415 (see pJAK plasmids in Table 1) and tested for expression of the *inc* phenotype, as described above. In each case 50 transconjugants were tested. Loss of pTAV3 was observed in all tested transconjugants carrying the following pRK415-derivatives: pJAK574 (carries module C), pJAK283 (carries B + C) and pJAK2345 (carries A + B + C) (data not shown). The lack of the autonomous form of pTAV3 (verified by in-gel lysis and DNA electrophoresis) was accompanied by loss of the ability of transconjugants to grow on minimal media (a feature associated with pTAV3), which excludes the possibility of pTAV3 persisting in the form of a cointegrate within a chromosome.

The module C is thus the smallest DNA fragment able to express *inc* phenotype, therefore we consider it *inc2* (Fig. 2).

3.4. Functional analysis of inc2

To determine whether the identified *inc2* contains the partitioning site, functional analysis of this region was performed, including (i) transcriptional silencing and (ii) mutational analysis.

(i) In a few cases, the overproduction of ParB-like proteins have been shown to cause destabilization of plasmids carrying a partitioning site located a certain DNA context (e.g., Kim and

Wang, 1999; Kusukawa et al., 1987; Rodionov et al., 1999). It is believed that the basis of such destabilization lies in transcriptional silencing of the *rep* genes by ParB, thus precluding plasmid replication.

To check whether this phenomenon could be observed in the case of *inc2* (module C), the sequence was cloned into the silencing reporter vector pGB2 (Sp^r), giving plasmid pPDB11. The multiple cloning site (MCS) of pGB2 is located in close proximity to the promoter of the *repA* gene (coding for replication initiator). The source of ParB protein was plasmid pPDB30/ParB (Ap^r), in which the PCR-amplified *parB* gene (sequences of the nucleotide primers used are given under Section 2) was cloned into vector pQE30. Overexpression of the protein [ParB (His_6)] was verified by SDS–PAGE (data not shown).

We were unable to obtain transformants carrying both pPDB30/ParB and pPDB11, which was most probably due to strong destabilization. In a control experiment, plasmid pGB2 was stably maintained, when grown without Sp selection, both in the absence and in the presence of plasmid pPDB30/ParB (overexpressing ParB). This result strongly suggested that the *inc2* sequence (module C) contains a ParB binding site(s), which constitutes the partitioning site of pTAV3. To confirm this speculation the ParB protein was purified and used together with the PCR amplified modules in an electrophoretic mobility shift assay (EMSA) as described under Section 2. As expected, retardation of migration of ParB–DNA complexes was observed exclusively for DNA fragments carrying module C (Fig. 3).

(ii) To confirm the importance of *inc2* in plasmid partitioning, mutational analysis was also performed. Initially a 4.1 kb *Bam*HI–*Kpn*I *par*-containing restriction fragment of pTAV3 (Fig. 1) was cloned into vector pABW3, which carries the replicator region of a composite plasmid pTAV1, which is unstable in paracocci (approximately 4% of cells contain pABW3 after 30 generations of growth in non-selective conditions). The derived plasmid (pABW3/par) was as stable as pDBV54 (containing a complete basic replicon of pTAV3), when tested in pTAV1-less *P. versutus* UW400, which

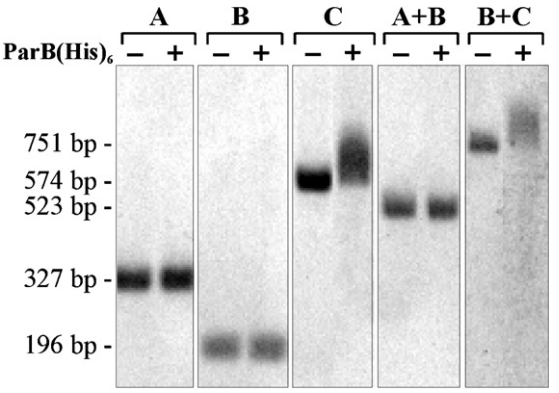

Fig. 3. EMSA demonstrating the binding of ParB(His_6 to module C (*inc2*). PCR amplified DNA fragments carrying modules A, B, C, A + B, and B + C were incubated in the absence (−) or presence (+) of purified ParB(His_6 protein as described under Section 2. Samples were resolved in 2% agarose gel, followed by transfer to a nylon membrane. DNA was detected by hybridization with DIG-labeled PCR amplified DNA fragment carrying A, B, and C modules.

confirmed the presence of all genetic information necessary for stabilization.

Deletion of the terminal 870 bp *Bam*HI–*Nde*I fragment generated in pABW3/par (Fig. 2B; containing modules A, B, and a part of C) completely abolished the plasmid stabilizing activity of this region (4% of tested cells carried the plasmid after approximately 30 generations of growth in non-selective conditions), while deletion of a 459 bp *Bam*HI–*Eco*47III fragment (Fig. 2B; containing module A and a part of module B) did not affect stability (no plasmid-less cells detected). In addition we found that disruption of the module C sequence by insertion of 4 bp within *Nde*I site reduced the stability of the thus mutated plasmid (35% cells carried the plasmid). This confirmed the crucial role of the sequence adjacent to *parB* (module C) in proper functioning of the partitioning system.

The above results suggested that the *Nde*I restriction site might be located within the ParB binding sequence. Its disruption may preclude binding of the ParB protein and consequently prevent its stabilizing activity. It is also possible that the observed phenotype might result from the increased size of the spacer sequence between the putative ParB binding sites. The only repeated

sequences present in module C (two 12 bp repeats) are situated on either site of the *Nde*I site (Fig. 2). It is noteworthy that the partitioning site of the *repABC*-type replicon of a composite plasmid pTAV1 (co-residing with pTAV3), is composed of two repeated sequences (non-homologous to those of pTAV3), which constitute the binding site for the ParB-like RepB protein (Bartosik et al., 2001).

The tandemly repeated sequences (module A), resembling the partitioning site of plasmid F (12 directly repeated sequences; Lane et al., 1987), appeared not to be crucial for partitioning. However, the possibility that they may constitute the site of binding for, as yet unidentified, accessory host proteins could not be excluded (as shown for IHF in the case of the *par* region of the P1 prophage in *E. coli*; Funnell, 1988).

3.5. Analysis of incompatibility of inc regions of pTAV3 with megaplasmids of P. pantotrophus

In previous studies we found that the origin of replication of pTAV3 can be activated by the trans-acting factor(s) delivered by megaplasmid pKLW2, harbored by a phylogenetically related *P. pantotrophus* DSM 11073 (Bartosik et al., 2002a). This showed that the replication systems of both plasmids are closely related. To check whether their complete basic replicons are functionally related we performed incompatibility analyses. For this purpose we introduced plasmids pDBV51 and pDBV56 [carrying the previously described *inc1* (Bartosik et al., 2002a) and *inc2* of pTAV3, respectively, cloned in a broad host range vector pCM66 compatible with the natural replicons of *P. versutus*] into four strains of *P. pantotrophus* (DSM 11072R, DSM 11073R, DSM 65R, and LMD 82.5R), carrying megaplasmids of different size (Fig. 4, lanes 1, 3, 5, and 7, respectively). It appeared that the introduced plasmids displaced the residing pWKS3 (DSM 11072R), pKLW2 (DSM 11073R), pHG16-b (DSM 65R), and pPAN2 (LMD 82.5R) (Fig. 4, lanes 2, 4, 6, and 8, respectively). Thus both *inc* regions of pTAV3 express incompatibility towards the megaplasmids of *P. pantotrophus*.

The presented data (together with our previous work concerning the replicator region of pTAV3; Bartosik et al., 2002a) represent a functional characterization of the basic replicon of megaplasmid/mini-chromosome pTAV3. The presence of pTAV3-like basic replicons within one chromosome of *R. sphaeroides* (cII; 943 kb) as well as within megaplasmids of *P. pantotrophus* shows that replicons of this kind are successful in maintaining large replicon genomes. So far little is known about the megaplasmids of *P. pantotrophus* (no DNA sequences are available). In all cases we observed that the loss of the megaplasmids resulted in a similar phenotypic effect (inability to grow on minimal media), which has previously been observed in pTAV3-less cells of *P. versutus* (Bartosik et al., 2002a). Together, our results show

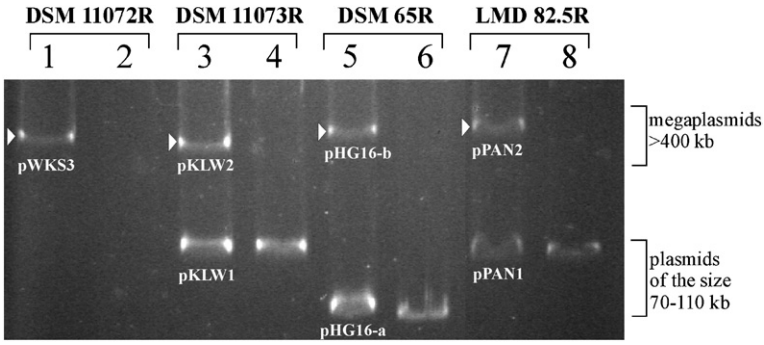

Fig. 4. DNA electrophoresis (after in-gel lysis) of four strains of *P. pantotrophus* (DSM 11072R, DSM 11073R, DSM 65R, and LMD 82.5R). Lanes 1, 3, 5, and 7—wild-type strains; lanes 2, 4, 6, and 8—megaplasmid-less derivatives obtained after the introduction (in a compatible broad-host-range vector pCM66) of *inc2* of pTAV3. Analogous results were obtained with *inc1* of pTAV3 (data not shown). Plasmid and megaplasmid designations are given below the respective DNA bands.

that pTAV3 and the megaplasmids of *P. pantotrophus* not only belong to the same incompatibility group of related replicons but they also seem to carry similar sets of, yet to be identified, housekeeping genes.

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