Molecular and functional analysis of pTAV320, a *repABC*-type replicon of the *Paracoccus versutus* composite plasmid pTAV1

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The second replicator region of the native plasmid pTAV1 of *Paracoccus versutus* has been identified thus proving the composite nature of this replicon. The minimal replicon designated pTAV320 (4.3 kb) was cloned and sequenced. pTAV320 encodes three putative proteins – RepA, RepB and RepC. This replicator region shows strong structural and functional similarity to *repABC*-type replicons found in several *Agrobacterium* and *Rhizobium* plasmids. The origin of replication appears to be localized within the coding sequence of the *repC* gene. RepC was shown to be essential for replication. RepA and RepB were necessary for stable maintenance of the plasmid, which implies a role in active partitioning. The presence of the complete sequence of pTAV320 (in its non-replicative form) could stabilize *in cis* pTAV202, a mini-replicon derived from the other pTAV1 replicator region. Deletions introduced into the *repC* gene abolished the ‘stabilizing’ activity of pTAV320, suggesting that the centromere-like sequence, necessary for partitioning, might be localized within this gene. The two replicator regions of pTAV1 (pTAV320 and pTAV202) expressed incompatibility towards the parental plasmid but were compatible *in trans* in *P. versutus* cells. The pTAV320 replicon can be maintained in several *Paracoccus*, *Agrobacterium*, *Rhizobium* and *Rhodobacter* strains in addition to *P. versutus*.

Keywords: *Paracoccus versutus*, pTAV1 composite plasmid, *repABC* replicon, plasmid replication and stability, minimal replicon

INTRODUCTION

Paracoccus versutus, previously *Thiobacillus versutus*, is a Gram-negative, facultatively chemolithoautotrophic soil bacterium belonging to the α subclass of the *Proteobacteria*. *P. versutus* UW1 harbours a large (107 kb), low-copy-number, cryptic plasmid, pTAV1 (Bednarska *et al.*, 1983), which is stably maintained in its bacterial host. The replication system of this plasmid has been identified by construction of a mini-replicon, pTAV202 (Bartosik *et al.*, 1995), and its shortened form pTAV203 (Bartosik *et al.*, 1997a), which confers all functions sufficient for autonomous replication in *P. versutus*. pTAV203 carries a 3.1 kb *SphI*–*PstI* restriction fragment of pTAV1 linked to the Km^r cassette derived from pUC4-K. The nucleotide sequence of this replicator region (GenBank accession no. U42228) revealed the

presence of two genes of the same transcriptional polarity encoding two putative proteins: RepC' (45 kDa) and RepX (26 kDa). Further analysis of pTAV203 showed that under certain conditions the *repC'* gene itself was able to provide replication function (Bartosik *et al.*, 1997b). Therefore, this proved that RepC' is the major replication initiator protein and that the origin of replication is located along its coding sequence.

The replication system of pTAV203 appears to be related to several plasmids commonly found in Gram-negative soil bacteria. RepC' shares statistically significant homology with the RepC replication protein of pRmeGR4a of *Sinorhizobium meliloti* (Mercado-Blanco & Olivares, 1994) and RepC of *repABC*-type replicons, e.g. pRiA4b of *Agrobacterium rhizogenes* (Nishiguchi *et al.*, 1987) and pRL8J1 of *Rhizobium leguminosarum* (Turner & Young, 1995). Despite the homology of RepC proteins, no other structural similarities between pTAV203 and other known replicons were found.

The GenBank accession number for the sequence reported in this paper is U60522.

The mini-replicon pTAV203 is not stably maintained in *P. versutus* cells. The stable maintenance of pTAV202 or pTAV203 and probably entire pTAV1 depended on a specific 14 kb *EcoRI*–*BclI* restriction fragment of the parental plasmid. This fragment when introduced *in cis* could fully stabilize pTAV203, but also carried a second replication system of pTAV1. Although it is suggested that many large plasmids are composite replicons (Helinski *et al.*, 1996), few examples have been described. These are mainly *Escherichia coli* plasmids belonging to the IncF incompatibility groups (Bergquist *et al.*, 1982) and the *Streptococcus* plasmid pAM α 1 (Perkins & Youngman, 1983).

We report here results of an experimental approach designed to characterize the second replicator region of pTAV1, included in the mini-replicon pTAV320. Molecular analysis of both replication systems will enable the study of possible interactions between them in the parental composite plasmid pTAV1.

METHODS

Bacterial strains, plasmids and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. Cultures were grown in Luria broth (LB) (Sambrook *et al.*, 1989) at 30 °C (*P. versutus*, *Agrobacterium tumefaciens* and *Rhodobacter sphaeroides*) or 37 °C (*E. coli*) and in TY medium (Beringer, 1974) at 30 °C (*Rhizobium etli* and *Rhiz. leguminosarum*). Antibiotics included in the media were used at the following final concentrations ($\mu\text{g ml}^{-1}$): kanamycin, 50; streptomycin, 100 for *Rhiz. etli* and 400 for *Rhiz. leguminosarum*; rifampicin, 50; tetracycline, 1 for *Rhod. sphaeroides*, 3 for *P. versutus*, 5 for *Rhiz. leguminosarum*, 10 for *Rhiz. etli* and 20 for *E. coli*.

DNA manipulations. Plasmid DNA was isolated according to Birnboim & Doly (1979) and purified by CsCl/ethidium bromide gradient centrifugation. Cloning experiments, digestion with restriction enzymes, ligation, treatment with the Klenow fragment of DNA polymerase I and agarose gel electrophoresis were conducted in accordance with standard procedures as described by Sambrook *et al.* (1989). All enzymes were purchased from either Promega or Boehringer Mannheim. DNA restriction fragments were purified from agarose with the DNA-Gel-Out kit (DNA Gdansk). For Southern hybridization (Sambrook *et al.*, 1989), DNA probes were labelled with digoxigenin (Boehringer Mannheim). Hybridization and visualization of hybridization products were carried out as recommended by the supplier.

PCR amplification. PCR was used to amplify the *repC* gene. Oligonucleotides were synthesized by Universal DNA. The 5' oligonucleotide contained the nucleotide sequence of the amino-terminus of RepC (including a putative ribosome-binding site), including a *PstI* restriction endonuclease site (underlined): 5' AACTGCAGCGTGAATCACATCATGG-AGTA 3'. The 3' oligonucleotide contained the nucleotide sequence encoding the carboxyl-terminus of RepC, including a *PstI* restriction site (underlined): 5' AACTGCAGGAGAC-TTAACAGCTGTTATG 3'. The amplification was performed in Thermocycler Hot-Shot 18 (DNA Gdansk) using the above synthetic oligonucleotides, *Thermus aquaticus* polymerase (DNA Gdansk) and pTAV1 as a template DNA. The amplification cycle used was 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 2 min; the

last cycle was followed by an additional extension step of 5 min.

DNA sequencing and analysis. The nucleotide sequence was determined in the DNA Sequencing and Oligonucleotides Synthesis Laboratory, Institute of Biochemistry and Biophysics, Polish Academy of Science, using a terminator sequencing kit and automatic sequencer (Alf Pharmacia). The two *PstI* fragments of pTAV320 (2.1 and 2.2 kb) were cloned into *E. coli* pBGS18 vector and the inserts were sequenced starting with universal and reverse primers and then with primers complementary to the previously determined sequence. Sequence analysis and comparison searches through the databases were performed with TFASTA, BLAST and GAP (Wisconsin Genetics Computer Group Sequence Analysis Software Package; GCG version 8.1). The alignment of the sequences was performed with the PILEUP program (GCG software). Phylogenetic analysis of RepC sequences was performed with the neighbour-joining method included in the PHYLIP package (Felsenstein, 1993).

Electroporation and transformation. Electroporation was carried out at 2500 V, 25 μF and 200 Ω (for *E. coli*) or 400 Ω (for *P. versutus*, *Paracoccus denitrificans* and *A. tumefaciens*) in a gene pulser apparatus (Bio-Rad) according to a modified Bio-Rad procedure (Wlodarczyk *et al.*, 1994). Electro-transformants were selected on solidified LB medium supplemented with appropriate antibiotics. Competent cells of *E. coli* TG1 were prepared and transformed as described by Kushner (1978).

Triparental mating. The overnight cultures (spun down and washed twice to remove antibiotics) of the donor strain *E. coli* TG1 carrying the mobilizable recombinant vector based on pABW2 (Bartosik *et al.*, 1997b), suitable recipient strain and *E. coli* DH5 α carrying the helper plasmid pRK2013 were mixed at a ratio 1:2:1. One hundred microlitres of such a mixture was spread on a plate of solidified TY or LB medium, depending on the recipient strain. After overnight incubation at 30 °C the bacteria were washed off the plate and suitable dilutions were plated on selective media containing rifampicin or streptomycin, selective markers of the recipient strains, and other appropriate antibiotic (tetracycline or kanamycin) to select transconjugants. Spontaneous resistance of the recipient strains to kanamycin and tetracycline was undetectable under these experimental conditions.

Plasmid stability. Cultures of *P. versutus*, *Rhiz. etli*, *Rhiz. leguminosarum* and *Rhod. sphaeroides* containing plasmids to be tested were grown in LB medium supplemented with appropriate antibiotics. The late exponential growth phase cells were diluted 10^{-3} in LB medium without antibiotics and grown at 30 °C. Cultures were diluted 10^{-3} at 24 h intervals. Samples taken at each dilution step were plated on solidified LB medium for estimation of the number of generations. From these plates 200 colonies were tested with the use of a Km^r or Tc^r marker by replica plating. The retention of plasmids, after approximately 30 generations, was determined as the percentage of Km^r or Tc^r colonies.

Incompatibility testing. The incompatibility characteristics of two plasmids were examined by electroporation of the plasmid to be tested into recipient bacteria carrying the other plasmid. Transformants were selected for the incoming plasmid or, in the case of analysis of coexistence *in trans* of the two pTAV1 mini-replicons, for incoming and residing plasmids. The plasmid pattern of the transformants was verified by screening 10 colonies by a rapid alkaline extraction procedure and agarose gel electrophoresis.

Table 1. Bacterial strains and plasmids used in this work

Strain or plasmid	Relevant characteristics	Reference or source
Strains		
<i>Paracoccus versutus</i>		
UW1	Wild-type (first described as <i>Thiobacillus</i> sp. strain A2)	Taylor & Hoare (1969)
UW225	Rif ^r , pTAV1-less derivative of UW1	Bartosik <i>et al.</i> (1993)
<i>Paracoccus denitrificans</i> KL1	Wild-type, carries native pKL1 (100 kb)	Jordan <i>et al.</i> (1997)
<i>Agrobacterium tumefaciens</i> LBA1010	Carries native pTiB6S3 (195 kb)	Koekman <i>et al.</i> (1982)
<i>Rhizobium etli</i> CE3	Str ^r	Noel <i>et al.</i> (1984)
<i>Rhizobium leguminosarum</i> 1062	Str ^r	Hirsch <i>et al.</i> (1980)
<i>Rhodobacter sphaeroides</i> 2.4.1R	Rif ^r derivative of a wild-type 2.4.1 strain	Suwanto & Kaplan (1992)
<i>Escherichia coli</i>		
TG1	Host strain for recombinant plasmids	Sambrook <i>et al.</i> (1989)
DH5 α	Host strain for pRK2013	Hanahan (1983)
Plasmids		
pTAV1	Wild-type, cryptic, 107 kb	Bednarska <i>et al.</i> (1983)
pTAV110	Mini-replicon carrying 30 kb <i>EcoRI</i> fragment of pTAV1 plus Km ^r cassette (1.3 kb) derived from pUC4-K	Bartosik <i>et al.</i> (1995)
pTAV112	pTAV110 derivative with 596 bp <i>Bgl</i> III deletion covering part of <i>repC</i>	This work
pTAV202	Mini-replicon carrying 4 kb <i>Sall</i> – <i>Pst</i> I restriction fragment of pTAV1 plus Km ^r cassette derived from pUC4-K	Bartosik <i>et al.</i> (1995)
pTAV202T	Tc ^r derivative of pTAV202, Tc ^r cassette derived from pBR322	This work
pTAV203	Mini-replicon containing a 3.1 kb <i>Sph</i> I– <i>Pst</i> I fragment of pTAV202 plus Km ^r cassette derived from pUC4-K	Bartosik <i>et al.</i> (1997a)
pTAV202/18	Km ^r , pBGS18 carrying 4 kb <i>Pst</i> I fragment of pTAV202	Bartosik <i>et al.</i> (1995)
pTAV202/18/16	Km ^r , 16 kb <i>Bcl</i> I fragment of pTAV110 cloned into <i>Bam</i> HI site of pBGS202/18	This work
pTAV301	pTAV1 mini-derivative composed of 16.1 kb <i>Bcl</i> I fragment of pTAV110 plus Km ^r cassette derived from pUC4-K	This work
pTAV310	Mini-replicon containing 9 kb <i>Sall</i> fragment of pTAV301 plus Km ^r cassette derived from pUC4-K	This work
pTAV320	Minimal replicon composed of two <i>Pst</i> I fragments (2.1 and 2.2 kb) of pTAV1 plus Km ^r cassette derived from pUC4-K	This work
pTAV321	pTAV320 derivative with 32 bp <i>Bam</i> HI deletion in <i>repA</i>	This work
pTAV322	pTAV320 derivative with inserted Km ^r cassette (from pUC4-K) into <i>Pst</i> I site of <i>repB</i> gene	This work
pUC4-K	Source of Km ^r cassette	Vieira & Messing (1982)
pBGS18	Km ^r , cloning vector, <i>ColE1</i> origin of replication	Spratt <i>et al.</i> (1986)
pABW2	Tc ^r , cloning vector based on pBGS18, Tc ^r derived from Tn1721, <i>oriT</i> of RK2	Bartosik <i>et al.</i> (1997b)
pABW320	Tc ^r Km ^r , pABW2 derivative containing 5.6 kb non-replicative form of pTAV320 linearized by <i>Stu</i> I	This work
pABW202	Tc ^r , pABW2 derivative containing 4 kb <i>Pst</i> I replicator region of pTAV202	This work
pABW201	Tc ^r Km ^r , pABW202 derivative containing 4.3 kb <i>Bsu</i> 36I– <i>Bgl</i> III fragment of pTAV320	This work
pABW2022	Tc ^r Km ^r , pABW202 derivative containing 5.6 kb non-replicative form of pTAV320 linearized by <i>Stu</i> I	This work
pABW2023	Tc ^r Km ^r , pABW202 derivative containing 5 kb <i>Bgl</i> III fragment of pTAV320	This work
pABW2024	Tc ^r Km ^r , pABW202 derivative containing 5.6 kb non-replicative form of pTAV320 linearized by <i>Bsu</i> 36I	This work
pABW2/repC1	Tc ^r , PCR-amplified 1.3 kb fragment of <i>repC</i> gene cloned into <i>Pst</i> I site of pABW2 under control of P _{tac}	This work
pABW2/repC2	Tc ^r , pABW2 with cloned <i>repC</i> gene in opposite orientation to that in pABW2/repC1	This work
pRK2013	Km ^r , helper plasmid carrying RK2 <i>tra</i> genes	Ditta <i>et al.</i> (1980)

RESULTS AND DISCUSSION

Identification of the second replicator region of plasmid pTAV1

Previously we constructed two mini-replicons carrying a 30 kb *EcoRI* (pTAV110) or 4 kb *SalI*–*PstI* (pTAV202) restriction fragment of the plasmid pTAV1 (Bartosik *et al.*, 1995), and the appropriate form of the Km^r cassette (1.3 kb). The mini-replicon pTAV202 was not stably maintained in *P. versutus* cells, whereas pTAV110 was as stable as the parental pTAV1. After 30 generations of growth under non-selective conditions, 100% of cells tested carried pTAV110, while only 4% carried pTAV202. We cloned several restriction fragments of pTAV110 which were not included in pTAV202 and tested their influence *in cis* on this unstable replicon to identify the region responsible for stability. For this purpose we used the hybrid plasmid pTAV202/18 (Bartosik *et al.*, 1995), which carried the 4 kb replicator region of pTAV202 cloned into the multiple cloning site of the *E. coli*-specific vector pBGS18 and showed a similar degree of stability (2%) to pTAV202. Insertion of the 16.1 kb *BclI* restriction fragment of pTAV110 into pTAV202/18 (pTAV202/18/16; Fig. 1) resulted in stabilization of the plasmid, indicating that this DNA fragment encoded mechanisms which enabled the stable maintenance of pTAV202. Plasmid pTAV202/18/16 was digested with *KpnI* and religated to make a considerable deletion (13.6 kb) in the ‘stabilizing’ fragment. We found a class of *P. versutus* Km^r transformants carrying a plasmid

(pTAV301; Fig. 1) with a different restriction pattern than expected. Detailed restriction analysis showed that the plasmid was composed of the excised region of the putative ‘stabilizing’ fragment (equipped with the Km^r cassette). Therefore, pTAV1 carries a second separable replication system designated pTAV301 (13.6 kb). Hybridization analysis proved that both replicons (pTAV301 and pTAV202) are present on different *BclI* restriction fragments of the parental pTAV1 (as well as of pTAV110) and do not cross-hybridize (data not shown).

To identify the minimal region of pTAV301, the plasmid was digested with *SalI* and, after ligation, was electroporated into *P. versutus* UW225 (pTAV1[−]). We obtained Km^r transformants carrying the mini-replicon pTAV310 containing the 9 kb *SalI* fragment of pTAV301. A partial digestion of pTAV310 with *PstI*, followed by ligation and further electroporation, enabled selection of transformants carrying a 5.6 kb plasmid, designated pTAV320. This replicon was composed of two (2.2 and 2.1 kb) *PstI* restriction fragments (aligned as in pTAV310 and pTAV1) and a Km^r cassette. We did not succeed in obtaining a replicative form of the plasmid composed of a single *PstI* restriction fragment of pTAV320 and Km^r cassette. Further analysis suggested that pTAV320 might constitute a minimal replicon of a second pTAV1 replicator region. The construction of pTAV320 is shown in Fig. 1.

Detailed restriction analysis of pTAV203, pTAV320 and

Fig. 1. Construction of the minimal replicon pTAV320. See Results and Discussion for explanations. The regions that contribute to the next stage of construction are marked with dashed lines. The Km^r cassette is marked as a box. Only restriction sites important for construction are shown. The sites for *Bsu*36I and *Stu*I are given only for pTAV320. Plasmids are not drawn to scale.

Fig. 2. Localization of the two identified replicator regions on pTAV1. The enlargements show (a) a detailed restriction map of the 30 kb *EcoRI* restriction fragment carrying the two identified replicator systems of pTAV1 and (b) restriction map and molecular organization of pTAV203 (Bartosik *et al.*, 1997a) and pTAV320. The orientation of ORFs is indicated by arrows.

pTAV110 localized both replication systems on a single 30 kb *EcoRI* restriction fragment of pTAV1 (present in pTAV110) as shown in Fig. 2.

Nucleotide sequence of pTAV320

To examine the gene organization of the replicator region, the complete nucleotide sequence of the minimal replicon pTAV320 was determined. Double-strand sequencing of pTAV320 covering the whole 4338 bp sequence revealed the presence of three large, clustered ORFs, with identical transcriptional polarity, encoding proteins of approximately 35 (RepA), 36 (RepB) and 47 (RepC) kDa (Fig. 2b). With the use of TFASTA (GCG software), we found that the putative translation products of pTAV320 show a significant and very similar level of homology to corresponding RepA, RepB and RepC proteins of pTiB6S3 of *A. tumefaciens* (Tabata *et al.*, 1989), pRiA4b of *A. rhizogenes* (Nishiguchi *et al.*, 1987), pRL8JI of *Rhiz. leguminosarum* (Turner & Young, 1995), p42d of *Rhiz. etli* (Ramírez-Romero *et al.*, 1997) and pNGR234a of *Rhizobium* sp. NGR234 (Freiberg *et al.*, 1997). For example, the similarity/identity between pTAV320 and the corresponding translation products of pTiB6S3 were: RepA, 71/56%; RepB, 52/32%; and RepC, 61/37%. RepA and RepB also have some similarity with several proteins encoded by non-*repABC*-type replicons (e.g. F and P1 plasmids; Williams & Thomas, 1992) and involved in partitioning, which may suggest a role for RepA and RepB in pTAV320. In addition, RepB shows similarity with ParB, a chromosome-partitioning protein of *Caulobacter crescentus* (Mohl & Gober, 1997). RepC of pTAV320 shows statistically significant degrees of similarity with the essential replication proteins RepC of pRmeRG4a of *S. meliloti* (29% identity) and RepC' of pTAV203, the second pTAV1 replication system (27% identity).

In addition to the sequence similarities, these genes are

similar in size and structural organization in pTAV320 to those of other *repABC*-type replicons. Thus pTAV320 appears to be the first example of this type of replicon found outside *Agrobacterium* and *Rhizobium*. Phylogenetic analysis of RepC proteins from replicons from the genera *Agrobacterium*, *Rhizobium* and *Paracoccus* indicated that RepC of pTAV320 is the most divergent of this group (data not shown).

In all published *repABC*-type replicons there is a short, highly conserved intergenic sequence (*igs*) situated between the *repB* and *repC* genes. In the case of pRiA4b and pTiB6S3 this intergenic region was shown to express incompatibility towards the parental plasmid. It was suggested that the origin of replication (*oriV*) might be located within this region (Tabata *et al.*, 1989). The multiple alignments of the *igs* sequences of different *repABC*-type plasmids are shown in Fig. 3. Although the *igs* of pTAV320 is more divergent, all the conserved motifs are visible. The highest degree of identity was observed in two regions. One of them contains an A/T-rich sequence followed by a G/C-rich region (AAAAG-AAAAAGGCCCC), which in the case of pTAV320, pTiB6S3 and pNGR234a is identical. This motif is also present between ORF2 and RepC in pRmeGR4 of *S. meliloti* and in the intergenic region between RepX and RepC' of pTAV203, which are non-*repABC*-type replicons, but is less conserved in the latter one (ATAAGT-AAACCCCC). In all cases this motif was followed by a potential stem-loop structure. The second most conserved region of the *igs* of pTAV320 and other *repABC*-type replicons (TTTGCCT) was located upstream of the *repC* gene. The motifs present in the *igs* sequence might be involved in regulation of *repC* transcription or act as a target for as yet unidentified regulatory proteins which could be involved in incompatibility functions (Ramírez-Romero *et al.*, 1997).

As has been observed in this type of replicon, there are two purine-rich regions, one preceding *repA* and the other *repC*, analogous in sequence and position to the

Fig. 3. Multiple alignments of the intergenic sequences (*igs*) of different *repABC*-type replicons: pNGR234a of *Rhizobium* sp. NGR234; pRiA4b of *A. rhizogenes*; pTiB6S3 of *A. tumefaciens*; pRL8JI of *Rhiz. leguminosarum*; p42d of *Rhiz. etli*; and pTAV320 of *P. versutus*. Alignment of at least five identical residues is shown on a black background. Hyphens indicate gaps introduced to maximize the alignment.

ribosome-binding sites preceding translated reading frames of enteric bacteria (Shine & Dalgarno, 1975). No sequence homologous to the -35 or the -10 promoter regions of *E. coli* was found upstream of the genes. The lack of a typical terminator structure within the pTAV320 sequence suggests that *repA*, *repB* and *repC* might constitute a common transcriptional unit. Further transcriptional analysis will help to elucidate the organization of the three loci.

Host range

The host range for pTAV1 has not yet been determined for lack of a convenient means of its transfer. pTAV1 is neither conjugative nor mobilizable. Its large size, low efficiency of electroporation and restriction barrier in many bacterial species make the use of transformation or electroporation ineffective. We have previously shown that the host range of pTAV202 is not limited to *P. versutus* and it can be maintained in *P. denitrificans* KL1 and *A. tumefaciens* LBA1010 (Bartosik *et al.*, 1997a). We successfully electroporated pTAV320 into both the above strains. As with pTAV202, pTAV320 does not replicate in *E. coli*.

To overcome the restriction barrier, we constructed a convenient mobilizable hybrid plasmid, composed of an *E. coli*-specific mobilizable pABW2 vector and a 5.6 kb *StuI* restriction fragment carrying the replication system of pTAV320 together with a Km^r cassette. The hybrid plasmid, designated pABW320, was introduced via triparental conjugal mating to *Rhiz. etli* CE3, *Rhiz. leguminosarum* 1062 and *Rhod. sphaeroides* 2.4.1R. This shows that pTAV320 is not a narrow-host-range replicon and can be maintained in several genera belonging to the α subclass of the *Proteobacteria*. pTAV320 (or pABW320) showed high levels of stability (92–100%) in all host strains tested and similar to that in *P. versutus* UW225. The preliminary data for pABW202 suggest the same host range for both pTAV1 mini-replicons.

Functional characterization of pTAV320

Plasmid replication. It was suggested that in the *repABC*-type replicons, *repC* encodes the main replication protein. Mutations introduced into this gene completely abolished the ability to replicate (Tabata *et al.*, 1989). The same mutational effect was observed in the case of pTAV320. It was not possible to obtain a replicon with a deletion of the 596 bp *Bgl*II fragment covering a terminal part of the *repC* gene (Fig. 2b), while mutations generated in *repA* or *repB* did not prevent replication. This proves that neither RepA nor RepB are indispensable for the initiation of replication. Deletion of a 32 bp *Bam*HI fragment of *repA* of pTAV320 (pTAV321) or insertion of the 1.3 kb Km^r cassette into the *Pst*I site of *repB* (pTAV322) affected stability of the mutated replicon. The stability of pTAV321 or pTAV322 was much reduced, and only 8 or 17% of the bacterial population, respectively, carried the plasmids (after 30 generations of growth under non-selective conditions), while pTAV320 was stably maintained (98%) in *P. versutus* cells. A similar mutational effect was previously observed for *repA* or *repB* genes of pRiA4b or pTiB6S3 (Tabata *et al.*, 1989).

We have previously shown that in pTAV202 the point of replication initiation (*oriV*) is located within the *repC'* gene. Because RepC' of pTAV202 and RepC of pTAV320 show homology and appear to play a similar role in replication, we assumed that *oriV* might be similarly located in pTAV320. To investigate this hypothesis, the 1.2 kb fragment carrying only the coding region for RepC with a putative ribosome-binding site was amplified by PCR and the fragment cloned, in two orientations (pABW2/repC1 and pABW2/repC2), into the *Pst*I site of pABW2. The resulting transformants were used as donors in triparental mating. We obtained Tc^r transconjugants in *P. versutus* UW225 only with pABW2/repC1, in which *repC* was placed under transcriptional control of the pABW2 *lac* promoter. This confirmed that RepC is sufficient for the initiation of replication and proved that *oriV* is located within its coding sequence. The sequence similarity between RepC and RepC' of pTAV1 and localization of *oriV* within

Fig. 4. Sequence and localization of the A/T-rich region of the *repC* gene of pTAV320. (a) A+T profile of the *repC* gene (1211 bp). (b) Sequence of the region containing the putative origin of replication. Horizontal arrows indicate 4 bp repeats.

both genes suggest a close relationship between these replication systems.

Within the central region of the *repC* sequence we found a 97 bp A/T-rich region of 63% A+T (compared to 39% overall pTAV320 DNA), containing three short (4 bp) repeats, followed by a potential stem-loop structure (Fig. 4). We speculate that the region extending from position 706 to 831 of the *repC* gene might contain the origin of replication. A similar region and structure, localized in an analogous position, has been previously suggested to form the *oriV* of megaplasmid pMOL28 of *Ralstonia eutropha* (*Alcaligenes eutrophus*) and it was suggested that in this case a transcriptional activation of the origin may occur (Taghavi *et al.*, 1996).

Partition functions. In the case of well-characterized partitioning systems it was possible to construct a specific cassette, encoding two proteins and a *cis*-acting centromere-like sequence, which could provide stability functions when cloned into other heterologous replicons (Williams & Thomas, 1992). To analyse the tentative partitioning nature of RepA and RepB we tested their influence *in cis* on pTAV202, the other unstable replicon of pTAV1. pTAV202 was lost during incubation under non-selective conditions and after 30 generations of growth only approximately 4% of the bacterial population harboured the plasmid. For the analysis we chose the mobilizable hybrid plasmid pABW202 (Table 1; Fig. 5), which enabled cloning in *E. coli* and efficient

conjugational transfer of the constructs into *P. versutus*. As a control the complete, non-mutated genome of pTAV320 linearized by *StuI* digestion was cloned into the blunted *HindIII* site of pABW202 (downstream of the 4 kb insert of pTAV202 to minimize a possible influence of *lac* promoter on expression of the cloned *rep* genes). As expected the derived pABW202 (carrying the two functional replicator regions of pTAV1) showed a similar level of stability to pTAV320 (Fig. 5). Assuming that the centromere-like sequence might be located along the highly conserved *igs* sequence we cloned the *Bsu36I*-*BglII* (4.3 kb) restriction fragment of pTAV320 carrying *repA*, *repB*, the complete *igs* region, 70 bp of *repC* and the *Km^r* cassette (enabling selection of recombinant clones). The resulting plasmid, designated pABW201, was not more stable than pABW202 itself (Fig. 5) and its stability was even decreased. This suggests that the cloned fragment did not contain a complete partitioning system (probably lacking the centromere-like sequence).

To test the possibility that the sequence required for partitioning might be localized along *repC*, we cloned into pABW202 the following fragments: (1) 5 kb *BglII* fragment from pTAV320 carrying *repA*, *repB*, *igs* sequence and part of the *repC* gene (779 bp); (2) the entire pTAV320 (5.6 kb) linearized with *Bsu36I* (which inactivates its replication activity). The resulting plasmids were designated pABW203 and pABW204, respectively. pABW203 showed a similar level of stability to pABW201, whereas pABW204 was more stable (51%) than pABW202 itself (1.5%). Thus only the presence of the whole sequence of pTAV320 (plasmid pABW204) was able to increase the stability of pTAV202. Construction and stability of all recombinant clones are shown in Fig. 5. In a further control experiment all the above-mentioned restriction fragments of pTAV320 were also cloned into pABW2, but only one, as expected, carrying the linearized genome of pTAV320 as a 5.6 kb *StuI* fragment (pABW320) was shown to be able to replicate in *P. versutus* (data not shown).

These results suggest that partition functions require the presence of an intact *repC* gene. To confirm the role of *repC* in partition we chose the fully stable pTAV110, a composite mini-replicon which carries the two replicator regions on a single *EcoRI* (30 kb) restriction fragment of pTAV1 (Fig. 2a). Introduction of the 596 bp *BglII* deletion into pTAV110 did not abolish the replication function of the resulting plasmid, pTAV112 (indicating that the second replicator region from pTAV202 carried out replication), but reduced its stability (<1%) to that of pABW203.

These results confirm that stable maintenance of the pABW202 recombinant plasmids, pTAV110 and probably pTAV1 is dependent on the presence of pTAV320, which carries the complete partitioning system. Our observations show that the coding sequence for the *repC* gene is important for plasmid maintenance and that *cis*-acting sequences for the origin of replication and the centromere-like sequence might be located within it.

Fig. 5. Analysis of the stabilizing activity of different fragments of the pTAV320 replicon on pTAV202 present in the recombinant plasmid pABW202. See text for explanations. The truncated *repC* gene is marked as a dotted arrow. Stability was determined as described in Methods.

Incompatibility of pTAV1 mini-replicons

Incompatibility of the two mini-replicons pTAV202 and pTAV320 towards the native pTAV1 was tested via electroporation of each mini-replicon into *P. versutus* UW1 carrying native pTAV1. The frequency of electroporation into this strain was 10^3 times lower than into UW225 (pTAV1-less strain) and none of the Km^r transformants carried the pTAV1 plasmid, which shows that both mini-replicons expressed strong incompatibility towards the residing parental plasmid. We also tested the possibility of the coexistence of the two mini-replicons *in trans* in *P. versutus* cells. For this purpose pTAV320 was electroporated into *P. versutus* UW225 carrying pTAV202T (a Tc^r derivative of pTAV202). The Km^r Tc^r transformants carried both plasmids, which were able to coexist during growth under non-selective conditions and were lost from the population at the rates characteristic for the individual plasmids (data not shown). These results indicate that both replicons are compatible *in trans* and suggest that incompatibility towards pTAV1 might be caused by the existence of common regulatory elements for the two replicator regions which are challenged by the incoming mini-replicons.

Some studies on the compatibility of pTAV1 mini-replicons with other plasmids present in *P. denitrificans* and *A. tumefaciens* have also been done. pTAV320 expresses incompatibility towards pTiB6S3 of *A. tumefaciens*, indicating a very close relationship between these plasmids which naturally occur in different bacterial genera. Interestingly, pTAV1 mini-replicons showed a different incompatibility behaviour towards the *P. denitrificans* pKL1 plasmid (~ 100 kb). pTAV320 expressed incompatibility towards pKL1 while pTAV202 could be maintained together with this plasmid (Jordan *et al.*, 1997). However, we found the mini-replicon pTAV110 to be fully compatible with pKL1, which suggests that in the presence of pKL1 the replication of the composite plasmid was carried by pTAV202. In this case the plasmid was fully stable, thus

confirming the role of the partitioning system of pTAV320 in the stable maintenance of pTAV110 (and probably entire pTAV1).

Concluding remarks

pTAV1 is a composite plasmid and carries, on a single *EcoRI* restriction fragment, two replicator regions able to form separate mini-plasmids. The two replicons express incompatibility towards the parental plasmid but are compatible when present *in trans* in *P. versutus* UW225 cells.

Sequence analysis of pTAV320 showed the presence of three ORFs similar in sequence and organization to *repA*, *repB* and *repC* found in *repABC*-type replicons of several *Agrobacterium* and *Rhizobium* plasmids. RepC was confirmed to be the major replicator protein and *oriV* was localized to the *repC* gene. RepA and RepB are necessary for stable maintenance of the plasmid. However, only the presence of the whole sequence of pTAV320 (in its non-replicative form) could stabilize pTAV202 *in cis*. This suggests a role for *repC* in plasmid partitioning.

pTAV320 can be maintained in several *Paracoccus*, *Agrobacterium*, *Rhizobium* and *Rhodobacter* strains but not in *E. coli*. Incompatibility of pTAV320 and pKL1 of *P. denitrificans* suggests that this type of replicon (*repABC*) might be widespread not only, as previously suggested, among species of *Agrobacterium* and *Rhizobium*, but also in other genera belonging to the α subclass of the *Proteobacteria*.

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