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Identification of the Partitioning Site within the *repABC*-Type Replicon of the Composite *Paracoccus versutus* Plasmid pTAV1

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The replicator region of composite plasmid pTAV1 of *Paracoccus versutus* (included in mini-replicon pTAV320) belongs to the family of *repABC* replicons commonly found in plasmids harbored by *Agrobacterium* and *Rhizobium* spp. The *repABC* replicons encode three genes clustered in an operon, which are involved in partitioning (*repA* and *repB*) and replication (*repC*). In order to localize the partitioning site of pTAV320, the two identified incompatibility determinants of this mini-replicon (*inc1*, located in the intergenic sequence between *repB* and *repC*; and *inc2*, situated downstream of the *repC* gene) were PCR amplified and used together with purified RepB fusion protein (homologous to the type B partitioning proteins binding to the partitioning sites) in an electrophoretic mobility shift assay. The protein bound only *inc2*, forming two complexes in a protein concentration-dependent manner. The *inc2* region contains two long (14-bp) repeated sequences (R1 and R2). Disruption of these sequences completely eliminates RepB binding ability. R1 and R2 have sequence similarities with analogous repeats of another *repABC* replicon of plasmid pPAN1 of *Paracoccus pantotrophus* DSM 82.5 and with centromeric sequences of the *Bacillus subtilis* chromosome. Excess RepB protein resulted in destabilization of the *inc2*-containing plasmid in *Escherichia coli*. On the other hand, the *inc2* region could stabilize another unstable replicon in *P. versutus* when RepA and RepB were delivered in *trans*, proving that this region has centromere-like activity. Thus, it was demonstrated that *repA*, *repB*, and *inc2* constitute a functional system for active partitioning of pTAV320.

Low-copy-number plasmids code for partitioning systems that ensure the correct distribution of plasmids within a bacterial population by precisely separating newly replicated copies into daughter cells at cell division. In general, these systems consist of three elements: (i) a *trans*-acting type A protein (ATPase) which autoregulates the operon and (ii) a *trans*-acting type B protein which binds to (iii) a *cis*-required partitioning site, which is thought to be analogous to the centromeres of eukaryotic chromosomes (27, 41). All three elements are indispensable for the proper functioning of these systems.

Although the partitioning systems are very often encoded close to plasmid replicator regions, they have been shown to function as independent cassettes that are able to stabilize (in *cis*) other, even unrelated replicons (30, 41). So far, there has been no evidence of structural association and coregulation of the two most important maintenance mechanisms for plasmids, replication and partitioning. The so-called *repABC*-type replicons seem to be an exception as the genes involved in the two processes are organized in an operon (5, 34).

The *repABC* family of replicons is widely distributed in bacteria belonging to the genera *Rhizobium* and *Agrobacterium* (32, 35). Several *repABC* replicons have been sequenced so far, including the root-inducing plasmids pRiA4b (29) and pRi1724 (28) of *Agrobacterium rhizogenes*; the tumor-inducing plasmids pTiB6S3 (39), pTi-SAKURA (38), pTiC58 (21), and pTiBo542 (containing in a tandem repeat two phylogenetically distinct *repABC*-type replicons) (31) of *Agrobacterium tumefaciens*; symbiotic plasmids pNGR234-a of *Rhizobium* spp. (13) and p42d of *Rhizobium etli* (34); cryptic plasmids pRL8JI of *Rhizobium leguminosarum* (40) and pAtC58 of *A. tumefaciens* (accession number AF283811); *Mesorhizobium loti* plasmids pMLa and pMLb, which coexist in *trans* (16); and plasmid pExo of *Sinorhizobium meliloti* (10). The sole representative of this class of plasmids that has been identified outside the *Rhizobiaceae* group is pTAV1 (mini-replicon pTAV320) of the soil bacterium *Paracoccus versutus* (5). pTAV320 appears to be the most divergent member of this family (5, 21, 32). pTAV1 is a composite plasmid, and in addition to pTAV320 it codes for a second replicator region (not included in the *repABC* class) that has been cloned in the form of mini-replicon pTAV202 (3, 7).

All of the *repABC*-type replicons have significant nucleotide and amino acid sequence similarities, and they have identical genetic organizations. They carry three clustered genes (*repA*, *repB*, and *repC*) that are transcribed in the same direction and a short, AT-rich, highly conserved intergenic sequence (*igs*) that is located between the *repB* and *repC* genes. The *repC* gene codes for the main replication initiator. Moreover, it has been suggested that the origin of replication is localized in the coding sequence of the *repC* gene (5, 32).

The *repA* and *repB* protein products have sequence similarities to proteins involved in partitioning of bacterial plasmids and their chromosomal homologues (14). Mutation analysis of the *repABC* replicons has demonstrated that mutations introduced into *repA* or *repB* drastically decrease plasmid stability (5, 34, 39), while parental plasmids are maintained in nearly 100% of the cells in the population even under nonselective conditions. The results of studies of Ramírez-Romero et al.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics[a]	Source or reference
P. versutus UW225	Rif^r; pTAV1-less derivative of wild-type UW1	6
E. coli strains		
TG1	Host strain for plasmids	37
DH5α	Host strain for pRK2013	37
M15	Km^r; host strain for pQE30 and pQE30/repB; carries repressor plasmid pREP4	Qiagen
Plasmids		
pTAV320	Km^r; 5.6-kb mini-replicon of pTAV1, composed of two *Pst*I fragments (2.1 and 2.2 kb) of pTAV1 plus Km^r cassette	5
pRK415	Tc^r; mobilizable broad-host-range vector; origin of replication (*oriV*) and origin of transfer (*oriT*) of RK2	17
pRK2013	Km^r; helper plasmid carrying RK2 *tra* genes	12
pRK415/M1	Tc^r; pRK415 derivative with 322-bp PCR fragment (*inc1*) (amplified with IGSL and IGSR primers) of pTAV320, containing 15 bp of *repB*, complete *igs*, and 95 bp of *repC* (coordinates 2497 to 2818)[b]	This study
pRK415/M2	Tc^r; pRK415 derivative with 394-bp PCR fragment (*inc2*) (amplified with ZACL and ZACR primers) of pTAV320, containing 139 bp of *repC* and 255 bp downstream of the gene (coordinates 3797 to 4190)	This study
PRK415/M3	Tc^r; pRK415 derivative with 2,208-bp *Pst*I fragment of pTAV320, containing proximal part of the replicon with *repA* and 680 bp of *repB* (coordinates 1 to 2208)	This study
pRK415/M4	Tc^r; pRK415 derivative with 1,323-bp *Bam*HI-*Pst*I fragment of pTAV320, containing 487 bp of terminal part of *repA* and 676 bp of *repB* (coordinates 881 to 2204)	This study
pRK415/M5	Tc^r; pRK415 derivative with 849-bp *Pst*I-*Bam*HI fragment of pTAV320, containing the promoter region and 446 bp of *repA* (coordinates 1 to 849)	This study
pRK415/M6	Tc^r; pRK415 derivative with 219-bp *Hin*dIII-*Bsu*36I fragment of pTAV320, containing 150 bp of *igs* and 69 bp of *repC* (coordinates 2574 to 2793)	This study
pRK415/M7	Tc^r; derivative of pRK415 with 370-bp *Pst*I-*Hin*dIII fragment of pTAV320, containing 307 bp of terminal part of *repB* and 63 bp of the intergenic region (*igs*) (coordinates 2204 to 2574)	This study
pRK415/M8	Tc^r; pRK415 derivative with 710-bp *Bsu*36I-*Bgl*II and 241-bp *Bgl*II-*Pst*I fragments of pTAV320, containing internal part of *repC* and terminal region of the mini-replicon, respectively (coordinates 2793 to 3503 and 4097 to 4338)	This study
pRK415/M11	Tc^r; pRK415 derivative with 1,143-bp *Bsu*36I-*Pvu*II fragment of pTAV320, containing 1,143 bp of *repC* (coordinates 2793 to 3936)	This study
pRK415/M20	Tc^r; pRK415 derivative with 2,204-bp *Pst*I-*Pst*I fragment of pTAV320, containing mutated *repA* (not in-frame insertion resulting from *Aat*II digestion and Klenow fragment end filling) and 676 bp of *repB* (coordinates 1 to 2204)	This study
pRK415/M21	Tc^r; pRK415 derivative with 594-bp *Bgl*II fragment of pTAV320, containing 432 bp of *repC* and 162 bp downstream of the gene (coordinates 3503 to 4097)	This study
pRK415/AB	Tc^r; pRK415 containing 2,737-bp *Bss*HII-*Bsu*36I restriction fragment of pTAV320 carrying *repA* and *repB* genes, *igs*, and 69 bp of *repC* (coordinates 56 to 2793)	This study
pTAV202	Km^r; mini-replicon of the second replicator region of plasmid pTAV1, containing 4-kb *Sal*I-*Pst*I restriction fragment of pTAV1 plus Km^r cassette; unable to replicate in *E. coli*	3
pABW1	Km^r; mobilizable cloning vector; ColE1 origin; *oriT* of RK2; unable to replicate in *P. versutus*	7
pABW3	Km^r; cloning-mobilizable *E. coli-P. versutus* shuttle vector composed of pABW1 and pTAV202	This study
pABW3/BGL	Km^r; pABW3 containing 594-bp *Bgl*II fragment of pTAV320 (coordinates 3503 to 4097)	This study
pQE30	Ap^r; plasmid for construction and expression of fusion His_6-tagged proteins; origin of ColE1	Qiagen
pQE30/repB	Ap^r; pQE30 containing 982-bp PCR-amplified (with primers REPBL and REPBR) *repB* gene of pTAV320 (coordinates 1528 to 2510)	This study
pREP4	Km^r; 3.7 kb; origin p15A; *lacI*^{q}	Qiagen
pGB2	Sp^r; cloning vector; pSC101 origin	11
pDBG1	Sp^r; pGB2 containing *inc2* region of pTAV320	This study
pDBG2	Sp^r; pGB2 containing *inc2* region of pTAV320 in the orientation opposite that of pDBG1	This study

[a] For plasmid derivative descriptions we used the nucleotide numbers of the sequence of pTAV320 as the coordinates.
[b] GenBank accession number U60522.

(33) also suggest that the protein products of the *repA* and *repB* genes of replicon p42d (*repABC*) bring about repression of the genes of the operon. Therefore, like the homologous proteins of other plasmid partitioning systems, they have important regulatory functions. It has also been shown that in the case of pTAV320 the nonreplicating form of a *repABC*-type replicon (with a disrupted *repC* gene) can stabilize an unstable plasmid in *cis* (5), which explicitly reflects the presence of a functional partitioning system in *repABC* replicons.

Previous studies have focused mainly on analysis of the replication functions of *repABC* replicons (5, 33). However, to fully understand the mechanism of action, it is also necessary to characterize the structural elements involved in plasmid partitioning. In this paper we describe the results of experi-

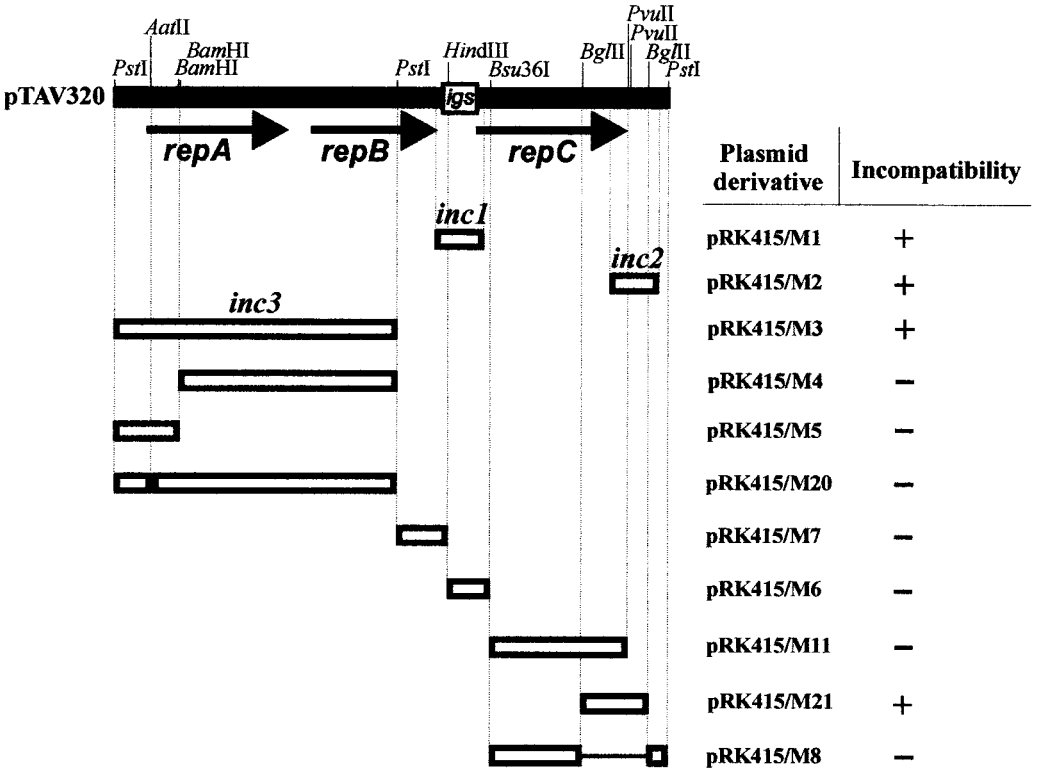

FIG. 1. Identification of incompatibility determinants (*inc*) of pTAV320. Only the restriction sites used to construct recombined plasmids are shown on the pTAV320 restriction map. The transcriptional orientations of the three open reading frames are indicated by arrows. The conserved intergenic sequence between *repB* and *repC* (*igs*) is enclosed in a box. The open boxes represent DNA restriction or PCR-amplified fragments of pTAV320 that were cloned into the pRK415 vector and tested for the presence of the *inc* regions; the designations of the pRK415 derivatives are on the right. For details concerning construction see Table 1. The lines joining the boxes indicate regions of pTAV320 removed by deletion. The smallest *cis*-acting regions of pTAV320 carrying identified incompatibility determinants are designated *inc1* and *inc2*. A plus sign indicates incompatibility and a minus sign indicates compatibility of pTAV320 with derivatives of pRK415 introduced into *P. versutus* UW225(pTAV320).

ments designed to localize the partitioning site of a *repABC*-type replicon (pTAV320), which together with *repA* and *repB* constitutes the functional partitioning system.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. All of the bacterial strains and plasmids used are listed in Table 1. Bacteria were grown in Luria-Bertani (LB) medium (37) at 30°C (*P. versutus*) or 37°C (*Escherichia coli*). The concentrations of antibiotics included in media were as follows: ampicillin, 100 μg/ml; spectinomycin, 50 μg/ml; kanamycin, 50 μg/ml; rifampin, 50 μg/ml; and tetracycline, 3 μg/ml for *P. versutus* and 20 μg/ml for *E. coli*.

DNA manipulations. Plasmid DNA was isolated by the method of Birnboim and Doly (8), and if necessary, it was 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 by using standard procedures, as described by Sambrook et al. (37). All enzymes were purchased from either Promega or Boehringer-Roche. DNA restriction fragments were recovered from agarose gels with a DNA Gel-Out kit (DNA Gdansk).

Plasmid construction. To identify the incompatibility determinant of pTAV320, a series of subclones containing selected restriction fragments or PCR-amplified fragments of the mini-replicon studied, cloned into vector pRK415, were constructed; detailed descriptions of the plasmids constructed are given in Table 1, and the construction scheme is shown in Fig. 1.

A mobilizable shuttle vector, pABW3, was constructed based on the *E. coli*-specific (nonreplicating in *P. versutus*) mobilizable vector pABW1 (*oriT* RK2) (4) and the *P. versutus*-specific (nonreplicating in *E. coli*) mini-replicon pTAV202 (7). Briefly, pABW1 was digested with *Mlu*I enzyme, which resulted in two restriction fragments, a 1.5-kb fragment containing the Km^r cassette and a 4.5-kb fragment containing all of the vector. Mini-replicon pTAV202 (Km^r) was linearized by *Sal*I digestion (at a unique restriction site flanking the kanamycin resistance cassette), blunt ended with the Klenow fragment of polymerase I, and ligated with a blunt-ended 4.5-kb *Mlu*I fragment of pABW1.

Electroporation and transformation. Electroporation was carried out at 2.5 kV, 25 μF, and 200 Ω (for *E. coli*) or 400 Ω (for *P. versutus*) with a Genepulser apparatus (Bio-Rad Laboratories) by using a modified Bio-Rad procedure (42). Electrotransformants were selected on solidified LB medium supplemented with the appropriate antibiotic. Competent cells of *E. coli* TG1 and M15 were prepared and transformed as described by Kushner (19).

Triparental mating. Overnight cultures (spun down and washed twice to remove antibiotics) of the donor strain *E. coli* TG1 carrying a mobilizable vector, the recipient strain *P. versutus* UW225, and *E. coli* DH5α carrying the helper plasmid pRK2013 were mixed 1:2:1. Then 100 μl of the mixture was spread on a plate containing solidified LB medium. After overnight incubation at 30°C, the bacteria were washed off the plate, and suitable dilutions were plated on selective media containing rifampin (selective marker for the recipient strain) and another antibiotic (tetracycline or kanamycin) to select transconjugants. Spontaneous resistance of the recipient strains to kanamycin and tetracycline was not detectable under these experimental conditions.

PCR amplification. The *repB* gene was amplified with primers REPBL (5′-AAGGATCCATGCCGTCAACGACGAGATC-3′) and REPBR (5′-AAGGATCCCAGTCTTCCTTGCGCT TCCA-3′) (the introduced *Bam*HI restriction sites are underlined). For amplification of the *inc1* and *inc2* regions the following pairs of forward and reverse primers were used: primers IGSL (5′-CGCAAGGAAG ACTGAGGAAG-3′) and IGSR (5′-GGCTTACCCGGAACAGATAC-3′) and primers ZACL (5′-CGTCGCTATGCTGGAACGCT-3′) and ZACR (5′-CCGC ACATTGGACTGGCTCA-3′). The minimal region carrying the R1 and R2

repeats was amplified with primers INC10 (5′-TGATGTCCCGGCGAGATGC T-3′) and INC11 (5′-CTCTCTTCTGCTACCGACGC-3′). Amplification was performed with a Hot-Shot 18 thermocycler (DNA Gdansk) by using the synthetic oligonucleotides described above, *Thermus aquaticus* polymerase (Perkin-Elmer), and pTAV320 as the template DNA. PCR products were separated by 1% agarose gel electrophoresis, extracted from the gel with a DNA Gel-Out kit (DNA Gdansk), and used for analysis.

Incompatibility testing. The incompatibility characteristics of two plasmids were examined by conjugational transfer of tester recombinant Tc^r plasmids of the pRK415M series into a recipient strain (*P. versutus* UW225) carrying pTAV320 (Km^r). Transconjugants were selected for the incoming and resident plasmids or, in case of strong incompatibility, only for the incoming plasmid. The plasmid patterns of transconjugants were verified by screening 10 colonies with a rapid alkaline procedure and agarose gel electrophoresis. The incompatibility behavior of pTAV320 (which coexisted in *trans* with plasmids belonging to the pRK415M series) was tested during growth for approximately 30 bacterial generations. The retention of pTAV320 was determined by determining the percentage of kanamycin-resistant colonies among 200 Tc^r clones (containing a pRK415-based plasmid).

Plasmid stability. The stability of plasmids during growth in nonselective conditions was tested as previously described (5). Briefly, stationary-phase cultures were diluted in fresh medium without antibiotic selection and cultivated for approximately 10, 20, and 30 generations. Samples taken after cultivation for 10, 20, and 30 generations were diluted and plated onto solid medium in the absence of selective drugs. Two hundred colonies were tested with the Km^r or Tc^r marker by replica plating. The retention of plasmids after approximately 30 generations was determined by determining the percentage of kanamycin- or tetracycline-resistant colonies.

Overproduction and purification of RepB$(His)_6$ protein fusion. For overexpression of the RepB$(His)_6$ protein, 800 μl of an overnight culture of *E. coli* M15 carrying a recombinant plasmid (designated pQE30/repB) was used to inoculate 40 ml of fresh LB medium with ampicillin (100 μg/ml) and kanamycin (50 μg/ml), and the resulting culture was grown at 37°C to an optical density at 600 nm of 0.8. The culture was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a concentration of 1 mM and incubated for an additional 3 h. The cells were harvested and resuspended in 50 mM phosphate buffer (pH 8) containing 1 M NaCl. The cells were sonicated and centrifuged for 20 min at 22,000 × *g* and 4°C. A sample of the supernatant of the crude extract was mixed with an equal volume of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, boiled for 5 min, and subjected to SDS-PAGE on a 12.5% polyacrylamide gel. The gel was stained with 0.5% Coomassie brilliant blue in 25% methanol–10% acetic acid. The protein extract was mixed and incubated with 1 ml of metal resin (Ni-NTA agarose; Qiagen) with gentle agitation for 1 h at 4°C. The supernatant was removed, and the resin was washed four times with 4 ml of washing buffer (50 mM phosphate buffer, 300 mM NaCl, 10% glycerol; pH 6). The RepB$(His)_6$ protein was eluted from the resin by using 1-ml portions of elution buffer (washing buffer with increasing imidazole concentrations [50 to 300 mM]). The purification was monitored by SDS-PAGE and Coomassie blue staining. The eluted fractions chosen were dialyzed against washing buffer in order to remove imidazole.

Immunological detection of RepB$(His)_6$. Total-protein extracts or purified RepB$(His)_6$ protein was subjected to electrophoresis on an SDS–12.5% PAGE gel. The gel was washed in transfer buffer (192 mM glycine, 20% methanol, 25 mM Tris-HCl; pH 8.3). The proteins were transferred to a nitrocellulose membrane with a Semi-Phor transfer cell (Hoefer) for 1 h at 50 V. The efficiency of transfer was verified by staining the proteins with Ponceau S dye (Sigma). The dye was washed out with water, and immunological detection with Penta · His antibodies (Qiagen) was performed as recommended by the manufacturer.

DNA labeling and DNA mobility shift assay. PCR-amplified fragments carrying *inc1* and *inc2* regions of pTAV320 (or PCR-amplified or restriction fragments of *inc2*) were labeled by using T4 polynucleotide kinase and [γ-^{32}P]dATP (30 μCi; Amersham) and incubated at 37°C for 30 min. An oligonucleotide coding for the R1/R2 sequence was created by labeling the single-stranded DNA (5′-TAACAGCTGTTAAGTCTC-3′) and annealing it with the complementary sequence in a buffer containing 5 mM $MgCl_2$, 20 mM Tris (pH 7.5), and 50 mM NaCl. A DNA binding reaction was performed at 37°C for 5 min by incubating 1 μl of purified labeled fragment (0.5 ng of DNA) with 2 μl of protein extract containing overproduced RepB$(His)_6$ or purified proteins [RepB$(His)_6$ or integration host factor (IHF)] and nonspecific competitor DNA (calf thymus DNA) in binding buffer (40 mM Tris-HCl [pH 8.0], 10 mM $MgCl_2$, 100 mM KCl, 1 mM dithiothreitol, 100 μg of bovine serum albumin per ml). The final volume of the reaction mixture was 20 μl. One microliter of loading dye (0.1% xylene cyanol, 50% glycerol) was added, and the samples were immediately loaded and separated on 5% acrylamide-bisacrylamide (82:1) gels in 0.05 M Tris-borate-EDTA buffer (pH 8.3) by electrophoresis for 1.5 h at approximately 10 V/cm^2. The gels were transferred to Whatman 3MM paper, dried, and exposed to Kodak X-OMAT film for 24 h at −80°C.

Sequence analysis. Sequence analysis was done with programs included in the Genetics Computer Group sequence analysis software package (GCG version 8.1). Comparison searches of databases were performed with the BLAST program, provided by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST).

RESULTS

Identification of incompatibility determinants of pTAV320. Two incompatible plasmids (carrying related replication or partitioning systems) cannot be stably maintained in a bacterial cell in the absence of selective pressure. In partitioning systems the *cis*-required partitioning sites are, most commonly, the incompatibility determinants (*inc*) (2). To identify the partitioning site of pTAV320, a preliminary analysis was performed to identify the *inc* regions of the mini-replicon. To do this, several selected restriction fragments or PCR products were cloned into the broad-host-range vector pRK415 (Tc^r; compatible with pTAV320) in *E. coli* TG1 and transferred by triparental mating into *P. versutus* UW225 carrying pTAV320 (Km^r). The presence of the *inc* region in the incoming pRK415-derived plasmid resulted in a loss of pTAV320 (see Materials and Methods for details).

A previous analysis of one of the *repABC* replicons (p42d) led to identification of two sequences coding for incompatibility determinants; one of these sequences is located in the intergenic segment (*igs*) between the *repB* and *repC* genes, and the other is located downstream of the *repC* gene (33). Therefore, we first attempted to analyze analogous regions of pTAV320, and we found that both PCR-amplified *igs* of pTAV320 (present in pRK415/M1 [Fig. 1]) and an amplified segment that included the region downstream of the *repC* gene (present in pRK415/M2 [Fig. 1]) carried incompatibility determinants. The sequences were designated *inc1* (322 bp) and *inc2* (394 bp), respectively (Fig. 1). A more detailed analysis performed with pRK415-derived plasmids carrying cloned restriction fragments that included the entire pTAV320 genome (the plasmids constructed are listed in Fig. 1; details of the construction are given in Table 1) led to identification of a third *inc* region (*inc3*) cloned in pRK415/M3 (Fig. 1). In the case of pRK415/M3 it seemed probable that the incompatibility observed was the result of the presence on this plasmid of a functional gene (with adjacent promoter region) coding for the regulatory protein RepA (the *repA* gene is also under control of the P*lac* promoter of the vector). Further analysis revealed that plasmids containing subcloned restriction fragments of the *inc3* region (pRK415/M4 and pRK415 M/5 [Fig. 1]) or the whole fragment, in which a small deletion (4 bp) resulted in a change in the *repA* reading frame (pRK415/M20 [Fig. 1]), were compatible with pTAV320. These results explicitly indicate that in this case the overproduced RepA protein caused destabilization of pTAV320 residing in *trans*. A similar observation was made for p42d (33).

As shown on Fig. 1, pTAV320 was also incompatible with pRK415/M21 (which contains a 594-bp *Bgl*II fragment of pTAV320 coding for the terminal part of the *repC* gene and a 163-bp region downstream of *repC*). Since the *inc2* region (present in pRK415/M2) includes the terminal part of the *repC*

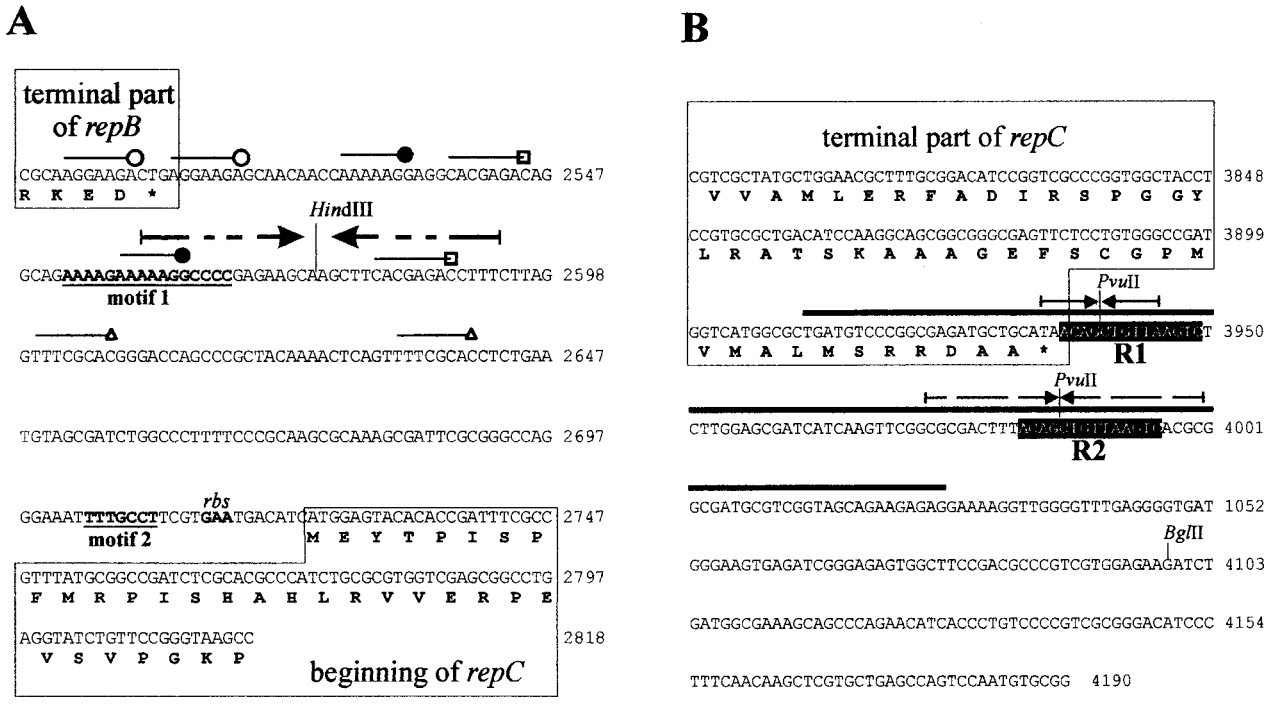

FIG. 2. Nucleotide sequences of the *inc1* (A) and *inc2* (B) regions of mini-replicon pTAV320. Several short repeated sequences identified in *inc1* are indicated by different lines. Motif 1 and motif 2, the two motifs of *inc1* sequences that are visibly conserved in the *igs* of *repABC* replicons, are indicated by boldface type and underlined. Two direct repeats of *inc2*, R1 and R2, are indicated by a black background. The region of *inc2* amplified with primers INC10 and INC11 and used for EMSA is indicated by a thick line over the sequence. The palindromic sequences of the *inc* regions are indicated by arrows. The terminal fragments of the *repB* and *repC* genes are enclosed in boxes. The amino acid sequences of the proteins are given below the nucleotide sequences. The *Hin*dIII, *Pvu*II, and *Bgl*II restriction sites important for the analysis are indicated. A presumptive ribosome binding site (rbs) is indicated. Coordinate numbers for the pTAV320 sequence are indicated on the right (accession number U60522).

gene (139 bp) and a sequence downstream of the gene (255 bp), whereas the terminal fragment of the gene (present in pRK415/M11 [Fig. 1]) does not express incompatibility with pTAV320, it seemed obvious that the incompatibility determinant had to be located downstream of *repC*, between the terminal codon of the gene and the *Bgl*II restriction site (163 bp) (Fig. 2B). The results obtained in further experiments confirmed this assumption.

Analysis of nucleotide sequences of the *inc* regions. A detailed analysis of the nucleotide sequences of the *inc* regions identified was carried out to identify potential structural elements analogous to those that occur in other plasmid partitioning sites that have been characterized (e.g., AT sequence enrichment, repeated and palindromic sequences). In the case of the *igs* sequence of pTAV320 (*inc1*) the G+C content was 52%, which is slightly different from the value determined for the entire mini-replicon (60%). However, several short stretches in which there was a high level of AT enrichment were distinguished in this sequence, as were several repeated sequences that were 7 to 8 bp long (some of the repeats occurred partially in the terminal part of the *repB* gene [Fig. 2A]). In the proximal part of *igs* two long imperfect palindromic sequences were found. It was observed that cleavage of the palindrome at the *Hin*dIII restriction site (Fig. 2A) completely eliminated the *inc* phenotype (Fig. 1) (pRK415/M6 and pRK415/M7). Although the *igs* sequence of pTAV320 is conserved less than the *igs* sequences in the other *repABC* replicons (5), two distinct conserved motifs were distinguished, as shown in Fig. 2A.

The G+C content of the *inc2* sequence is 60%. There are no areas with above-average A+T content. Also, this sequence does not contain any significant structural features except the two longest such features in the entire pTAV320 sequence, identical 14-bp repeated sequences (5′-ACAGCTGTTAAGT C-3′) that are separated by 33 bp (Fig. 2B). The first of these two repeats (designated R1) occurs directly after the *repC* gene (it begins in the termination codon of the gene), and the second (designated R2) lies 46 bp downstream of this gene (Fig. 2B). The palindromic sequences that are partially present in both direct repeat R1 and direct repeat R2 are shown in Fig. 2B.

Purification of RepB(His)$_6$ and determination of RepB binding site. As mentioned above, the partitioning type B proteins (RepB homologues) specifically bind to the corresponding partitioning sites. To determine the site of RepB interaction within pTAV320, the protein was purified and used in an electrophoretic mobility shift assay (EMSA).

(i) Purification of RepB(His)$_6$. For construction of the recombinant RepB protein, PCR was used to amplify the coding region of *repB* (see Materials and Methods). 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 *repB* gene and a vector sequence coding for six histidines (His$_6$ tag). The insert was sequenced to confirm that

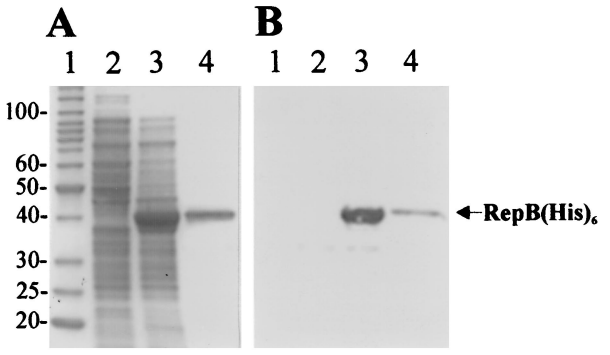

FIG. 3. (A) Coomassie blue-stained SDS-PAGE gel of overexpressed (in *E. coli* M15) and purified RepB(His)$_6$ protein. The gel was loaded with the following samples: protein molecular weight standards (lane 1) (the molecular masses of the proteins [in kilodaltons] are indicated on the left); crude cell lysate of strain M15(pQE30/repB) (lane 2); crude cell lysate of strain M15(pQE30/repB) after IPTG induction (lane 3); purified RepB(His)$_6$ protein (lane 4). (B) Western transfer of proteins visualized on the gel in panel A and immunodetection of RepB(His)$_6$ with Penta · His antibody.

no errors had been introduced during PCR. The resulting plasmid (pQE30/repB) was transferred into *E. coli* M15 carrying the repressor plasmid pREP4 (Table 1). IPTG induction resulted in a high level of expression of a protein (approximately 40 kDa) which was visualized by SDS-PAGE of the cell lysate of *E. coli* M15(pQE30/repB) (Fig. 3A, lane 3). This protein was not observed in a noninduced bacterial culture (Fig. 3A, lane 2). The protein was then purified on an Ni-NTA chromatographic matrix (Qiagen) (Fig. 3A, lane 4) as described in Materials and Methods. After SDS-PAGE and Western transfer, the RepB(His)$_6$ protein gave a positive reaction with Penta · His antibodies (Fig. 3B, lanes 3 and 4), thus proving its recombinant nature.

(ii) Determination of RepB binding site. For protein-DNA binding experiments the PCR-amplified 322-bp *inc1* DNA fragment and 394-bp *inc2* DNA fragment (Fig. 2) were ^{32}P labeled and used with cell extract containing overproduced RepB(His)$_6$ (or with the purified protein) for EMSA as described in Materials and Methods. As Fig. 4B shows, the RepB fusion protein bound in *inc2*. Two complexes having different electrophoretic mobilities (Fig. 4B, complexes I and II) appeared in the gel in a protein concentration-dependent manner. Complex 1 was preferentially formed at low Rep(His)$_6$ concentrations, whereas higher protein concentrations resulted in the formation of dominant complex II (Fig. 4B). This might have represented occupancy of two different target sites. In a control reaction similar amounts of *E. coli* M15(pQE30) cell extract were used (Fig. 4C, lanes 2 and 5). The lack of any complex formation indicated the specificity of RepB binding to the DNA fragment studied. No retardation of the *inc1* region was detected (Fig. 4A). No binding of purified *E. coli* IHF protein was observed in *inc1* and *inc2* (Fig. 4C, lanes 3 and 6). This protein is known to bind in the partitioning site of phage P1 (24).

As mentioned above, the *inc2* region contains two repeated sequences, R1 and R2 (Fig. 2B). Since analogous sequences frequently are sites to which plasmid proteins bind, we assumed that protein RepB might interact with a site in R1 and R2. To verify this, a small fragment of DNA (126 bp) carrying both repeats was PCR amplified (with primers INC10 and INC11 [Fig. 2B]) and used, together with purified protein, for EMSA. It appeared that RepB specifically bound the amplified fragment to form two analogous complexes, as in the case of the entire *inc2* fragment (Fig. 4D, lanes 4 to 6). Moreover, it was found that the amplified 162-bp DNA fragment was sufficient to express incompatibility with pTAV320 (data not shown).

The R1 and R2 sequences carry two *Pvu*II cleavage sites (the only *Pvu*II sites in the *inc2* fragment) (Fig. 2B). *Pvu*II digestion of the *inc2* fragment resulted in three restriction fragments that were 47, 142, and 205 bp long. As shown in Fig. 4D, lanes 1 to 3, cleavage of fragment *inc2* in R1 and R2 precluded binding of RepB, which strongly suggests that these sequences are the sites with which protein RepB interacts. To confirm this, an additional experiment was carried out, in which an oligonucleotide containing a single repeat was used as the substrate. In this case a shift of the DNA fragment (single complex) was observed, which explicitly confirmed that the R1 and R2 sequences are the recognition sites for the RepB protein (Fig. 4E, lanes 2 to 6).

Destabilization of *inc2*-containing plasmid by RepB. In the case of the partitioning system of P1 it was observed that overproduction of ParB (a RepB homologue) entailed destabilization (resulting from transcriptional silencing) of plasmid pGB2 carrying a partitioning site of phage P1 (*parS*) (23, 36). To check whether the pTAV320 sequence identified has similar properties, the *inc2* region was cloned in two orientations into multiple cloning site (MCS) pGB2 (Sp^r) (11), and the resulting plasmids (pDBG1, pDBG2) were introduced into *E. coli* TG1 containing RepB-overproducing plasmid pQE30/repB (Ap^r). The vector pGB2 is stably maintained in *E. coli* cells, both when it occurs alone and when it occurs in *trans* with compatible pQE30 or pQE30/repB. Plasmid pDBG1 or pDBG2 also stably coexisted with pQE30, whereas in the presence of pQE30/repB these plasmids became destabilized. Destabilization, regardless of the orientation of *inc2* in the plasmid, was so strong that it was impossible to obtain transformants of *E. coli* TG1 carrying both pDBG1 or pBDG2 and the RepB(His)$_6$ expression plasmid pQE30/repB. These results indicate that the presence of the overproduced protein results in destabilization of plasmids carrying *inc2*, which is most probably caused by transcriptional silencing of plasmid genes close to the cloned centromere.

Stabilization of *inc2*-containing plasmid by RepA and RepB. It has been observed repeatedly that unstable plasmids containing only a partitioning site can be actively partitioned if the two partitioning proteins of a given system are provided in *trans* (1, 20). To check whether the *inc2* sequence of pTAV320 is able to stabilize other replicons in *cis* (in the presence of *repA* and *repB* in *trans*), a two-plasmid system was constructed by using pRK415 (Tc^r), a broad-host-range vector, and the compatible mini-replicon pTAV202 (Km^r), which is able to replicate in *P. versutus* (3, 7). It was demonstrated previously that pTAV202 and pTAV320 which coreside in *trans* in *P. versutus* did not affect each other's maintenance (5).

In view of the strong restriction barrier that precludes introduction of plasmid DNA from *E. coli* into *P. versutus* by electroporation, for the purposes of this experiment the mobiliz-

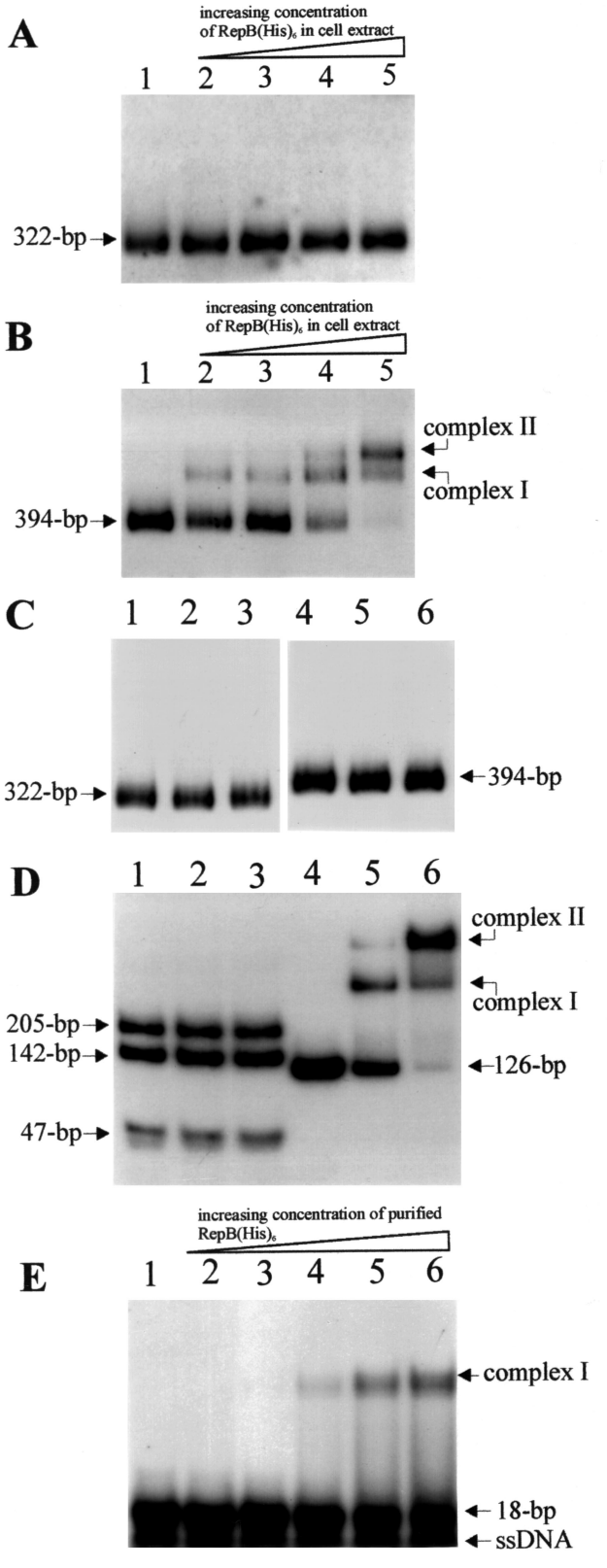

FIG. 4. Autoradiograph of EMSA results, showing the binding ability of RepB(His)$_6$ protein [present in crude protein extract of RepB(His)$_6$-overproducing *E. coli* M15 or purified] in ^{32}P-labeled *inc1* and *inc2* regions of pTAV320. (A and B) *inc1* fragment (A) and *inc2* fragment (B) incubated with different concentrations (2.5, 5, 10, and 20 μg/ml in lanes 2 to 5, respectively) of M15(pQE30/repB) crude protein extract. Lane 1 contained labeled fragment with no protein added. (C) *inc1* fragment (lanes 1 to 3) and *inc2* fragment (lanes 4 to 6) incubated (i) with M15(pQE30) crude protein extract (20 μg/ml) (lanes 2 and 5), (ii) with purified IHF protein (3 μg/ml) (lanes 3 and 6), and (iii) without protein (lanes 1 and 4). (D) The *inc2* fragment was digested with *Pvu*II (lanes 1 to 3) and incubated with 2.5 and 5 μg of purified RepB(His)$_6$ protein per ml (lanes 2 and 3, respectively). No protein was added to the reaction mixture in lane 1. The 126-bp PCR-amplified fragment of the *inc2* region (Fig. 2B) (lanes 4 to 6) was incubated with 2.5 and 5 μg of purified RepB(His)$_6$ protein per ml (lanes 5 and 6, respectively). No protein was added to the reaction mixture in lane 4. (E) An 18-bp oligonucleotide containing a single R1/R2 sequence was incubated with 0.5, 1, 2.5, 5, and 10 μg of purified RepB(His)$_6$ protein per ml (lanes 2 to 6, respectively). No protein was added to the reaction mixture in lane 1. The positions of the protein-DNA complexes and the sizes of the unbound probes are indicated on the right and left. ssDNA, single-stranded DNA.

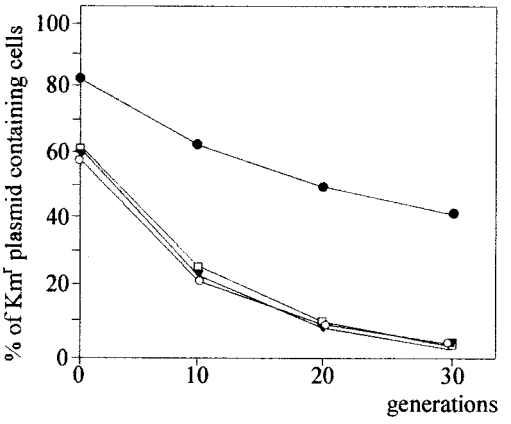

FIG. 5. Plasmid stability of pABW3 (Km^r) and pABW3/BGL coexisting in *trans* with compatible pRK415 (Tc^r) or pRK415/AB (carrying *repA* and *repB* genes of pTAV320) in *P. versutus* UW225 cells. Symbols: ▼, UW225 carrying plasmids pABW3 and pRK415; ○, UW225 carrying plasmids pABW3/BGL and pRK415; □, UW225 carrying plasmids pABW3 and pRK415/AB; ●, UW225 carrying plasmids pABW3/BGL and pRK415/AB. The data are averages based on three independent experiments.

able shuttle vector pABW3 (Km^r), containing a linear form of pTAV202 (5.3 kb) cloned into the *E. coli*-specific vector pABW1 (4), was constructed. The details are described in Materials and Methods. The resulting Km^r vector (8.5 kb) could be mobilized for conjugational transfer from *E. coli* to *P. versutus* and was able to replicate in both hosts.

The *inc2* region (present on a 594-bp *Bgl*II fragment of pTAV320 [Fig. 1]) was then cloned into the compatible *Bam*HI site (MSC) of pABW3, generating pABW3/BGL. At the same time, a 2.7-kb fragment of pTAV320, carrying the promoter region and the *repA* and *repB* genes, was cloned into MCS of pRK415; the resulting plasmid was designated pRK415/AB. Both plasmids were conjugationally transferred into *P. versutus* UW225, and the stability of pABW3/BGL coexisting in *trans* was determined. pABW3 itself is unstable in *P. versutus*; after 30 generations of growth in nonselective conditions, only approximately 4% of the cells carry the plasmid. The same degree of stability was observed in control experiments when pABW3 was maintained in *trans* with pRK415 or

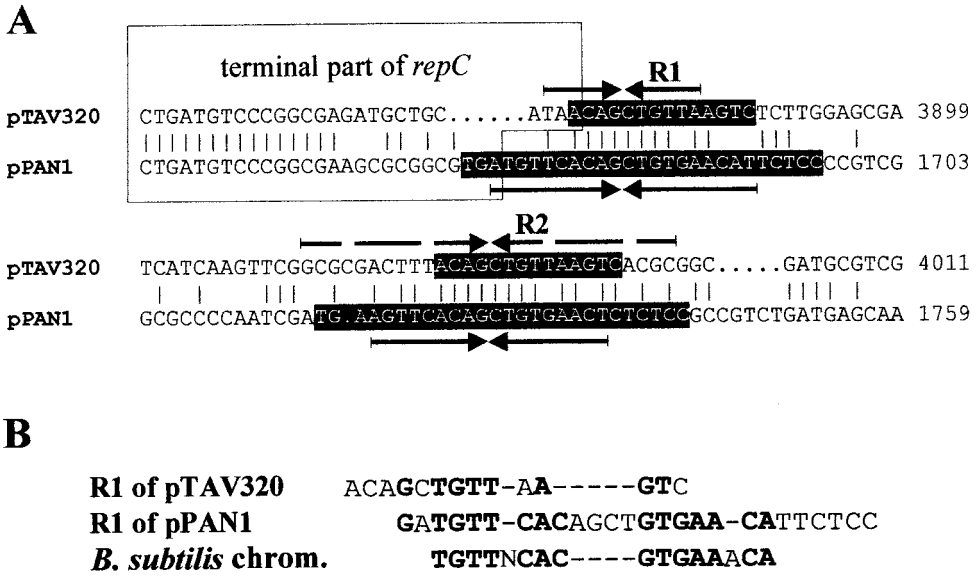

FIG. 6. (A) Alignment (BESTFIT; GCG software) of nucleotide sequences of the corresponding regions of *P. versutus* pTAV320 and the *repABC* replicator region of plasmid pPAN1 from *P. pantotrophus* DSM 82.5. The R1 and R2 repeated sequences are shown with a black background. The palindromic sequences are indicated by arrows. Terminal parts of the *repC* genes are enclosed in a box. Coordinates of the pTAV320 and pPAN1 sequences are indicated on the right. (B) Manual alignment of nucleotide sequences of R1 of pTAV320 and R1 of pPAN1 repeats and consensus centromeric sequence of the *B. subtilis* chromosome (chrom.) (22). Nucleotides common to two sequences at any position are indicated by boldface type. Dashes indicate gaps introduced to maximize the alignment.

pRK415/AB (Fig. 5). The *inc2* region, which was present in pABW3/BGL, did not affect the stability of the plasmid itself (as observed in the presence of pRK415) but could stabilize the plasmid (more than 40% of the cells carried the plasmid after 30 generations of growth in nonselective conditions) when *repA* and *repB* were provided in *trans* for pRK415/AB (Fig. 5). This confirmed that the region identified contains a functional *cis* required partitioning site and that RepA and RepB are partitioning *trans*-acting proteins.

Search for R1- and R2-like sequences in other *repABC* replicons. A comparative analysis of the nucleotide sequences of other *repABC* replicons did not reveal the presence (downstream of *repC*) of a sequence homologous to R1 and R2 of pTAV320. Moreover, no other equally long, significant, repeated sequences of any other type were found. The only exceptions were the replicons of the *A. tumefaciens* pTiSAKURA and pTiC58 plasmids, in which the terminal parts of the *repC* gene encode the first of two short (7-bp) repeated sequences (5′-GGTGATC-3′). These sequences are separated from each other (like R1 and R2 of pTAV320) by 33 bp.

Interestingly, repeated sequences of this type (length, 27 bp) have been localized in a newly identified replicon of the *repABC* type occurring on plasmid pPAN1 of *Paracoccus pantotrophus* DSM 82.5 (unpublished results); the partial nucleotide sequence has been deposited in the GenBank database under accession number AY033083. The locations of the repeated sequences of pPAN1 (5′-TGA[A/T]GTTCACAGCTG TGAAC[T/A][C/T]TCTCC-3′) are similar to the locations in pTAV320, and these sequences are similar to R1 and R2 (Fig. 6). The palindromic sequences present in both pPAN1 repeated sequences are shown in Fig. 6B. The repeated sequences of pPAN1 (and, to a lesser extent, the repeated sequences of pTAV320) are also similar to the centromere-like sequences of the *Bacillus subtilis* chromosome, which are the sites to which protein Spo0J (a chromosomal homologue in plasmid type B partitioning proteins) binds (22). An alignment of these sequences is shown in Fig. 6B.

DISCUSSION

In the present study we located the active partitioning site of the mini-replicon pTAV320, which belongs to the group of *repABC* replicons. Both the structure and the location of the partitioning site of pTAV320 differ from the structure and the location of the plasmid *par* loci that have been identified so far (27), in which sequences of this type are situated downstream of the gene coding for the type B partitioning protein (e.g., the *sopABC* loci of plasmid F and the *parABS* loci of phage P1) or in promoter regions of operons (e.g., the *parA* loci of plasmid R1 [9] and the *par* loci of plasmid pTAR). This sequence, which is a determinant of incompatibility (*inc2*), is located downstream of the gene coding for replication protein RepC (and thus downstream of the *repABC* operon). Two 14-bp repeated sequences, R1 and R2, as well as palindromic sequences, can be distinguished in the sequence and are the sites of binding of partitioning protein RepB.

Based on analogies with other active partitioning systems, it was postulated previously that the partitioning site of *repABC* replicons is located downstream of the *repB* gene, which is in the strongly conserved *igs* (*inc1*). The results of an analysis recently carried out by Ramírez-Romero et al. (33) seem to confirm this hypothesis. These authors observed that deletion of *igs* in p42d (*repABC* replicon) did not affect replication of the plasmid, whereas removal of the region directly behind the

repC gene completely eliminated replication of the mutated plasmid. In light of these results it seemed obvious that *igs* carries an incompatibility determinant involved in plasmid segregation (a partitioning site), whereas the region corresponding to *inc2* of pTAV320 contains the origin of replication.

Our previous results indicated, however, that *oriV* of pTAV320 is located in the coding sequence of the *repC* gene (5). A more detailed analysis of the *repC* sequence of all of the replicons of this group allowed workers to distinguish a region in these genes with an above-average A+T content that is characteristic of many plasmid *oriV* genes (32). In our studies we also determined that *inc2* of pTAV320 introduced into *P. versutus* on a plasmid that is not functional in this host is not able to replicate when replication protein RepC is delivered in *trans* (data not shown).

A region located downstream of *repC* seems to carry out important regulatory functions; it is crucial for both replication and active partitioning. Presumably, besides the partitioning site, there are other genetic elements (sites of interaction with as-yet-unspecified host- or plasmid-encoded factors) whose presence in *cis* is necessary for replication of the plasmid. It seems odd, however, that the nucleotide sequence of such a functionally important region is not conserved in all *repABC* replicons.

The role of *igs*, which carries the incompatibility determinant *inc1*, is puzzling. In this sequence conserved motif 1 (Fig. 2A) is present, which was also identified upstream of the *repC* genes of plasmids pRmeGR4a and pTAV202 (5, 26). These plasmids do not belong to the *repABC* family but encode phylogenetically distantly related homologues of the RepC protein. In all cases motif 1 is followed by a palindromic sequence, whose cleavage (in the case of pTAV320) completely eliminates the *inc* phenotype. The presence of this type of sequence in different plasmids carrying the *repC* gene suggests that *inc1* might be required for regulation of *repC* expression (5, 34), which in turn may be a factor that limits the frequency of initiation of replication. However, elucidation of the function of *igs* will require further detailed study.

In this study we observed that a plasmid carrying a partitioning site (*inc2*) in the presence of overproduced protein RepB of pTAV320 is subject to drastic destabilization in *E. coli*. This phenomenon was observed previously in analyses of the model active partitioning systems of plasmid F and phage P1 (18, 36). In both cases overproduction of type B partitioning proteins led to transcriptional silencing of genes adjacent to the centromere. In the case of plasmid pGB2 used by us, the promoter of the *repA* gene (coding for the protein that initiates replication) is close to the cloned *inc2* region. It seems that the cause of destabilization is blocked transcription of the *repA* gene resulting from binding of overproduced protein RepB, which in effect precludes replication of the plasmid. It is interesting that this effect is observed in *E. coli*, which is a host in which pTAV320 (like other *repABC* replicons) is not functional. Yet it is not known whether additional host-encoded factors are required for gene silencing in this case. However, the activity of the partitioning system of *repABC* replicons in *E. coli* has not been studied yet. It is probable that the host factors involved in plasmid partitioning are more conserved in different bacterial species than the host factors taking part in plasmid replication are. This has been confirmed by the observations of Yamaichi and Niki (43), who demonstrated the functionality of the chromosomal partitioning system of *B. subtilis* (a gram-positive bacterium) in *E. coli* cells (a gram-negative host).

Recent phylogenetic analyses of partitioning ATPases demonstrated that RepA of pTAV320 and the RepA proteins of the remaining *repABC* replicons are phylogenetically most closely related to the group of plasmid ATPases which includes SopA of plasmid F and ParA of phage P1 (14, 15). Interestingly, a BLAST search for RepB homologues resulted first in a long list of chromosomally encoded proteins that are homologues of type B plasmid partitioning proteins (data not shown). These results suggest that the active partitioning systems of pTAV320 and other replicons of the *repABC* type have a hybrid structure, since the active partitioning system of pTAV320 encodes a plasmid-specific ATPase and a chromosomal type of type B partitioning protein. However, it should be remembered that in view of their size, plasmids of the *Agrobacterium-Rhizobium* group harboring *repABC* replicons can be placed in the class of megaplasmids that occasionally are larger than the chromosomes of some bacteria. Moreover, as suggested by Palmer et al. (32), it is highly probable that replicons of this type can also occur on bacterial chromosomes.

The partitioning site of pTAV320 and that of an analogous sequence of another paracoccal replicon of this type, pPAN1, are similar (more so in the case of pPAN1) to the centromere-like sequences of the partitioning system of the *B. subtilis* chromosome (22). Similar sequences that are located in or close to a structural gene of a type B partitioning protein have also been localized in the chromosomes of many other bacteria, such as *Mycobacterium leprae*, *Mycobacterium tuberculosis*, *Streptomyces coelicolor*, *Borrelia burgdorferi*, and *Streptococcus pyogenes* (22). Although the remaining replicons of the *repABC* type do not have sequences homologous to R1 and R2, it should be kept in mind that pTAV320 and pPAN1 constitute a separate, evolutionarily distinct group of *repABC* replicons (21, 32; unpublished results). Therefore, they may be phylogenetically more closely related to chromosomal systems than to plasmid systems.

Interestingly, in the terminal parts of the mini-replicons of pTAV1 and pPAN1, downstream of the *repC* gene the beginning of a strongly conserved (at the nucleotide and amino acid sequence levels) incomplete open reading frame has been identified (data not shown). Its protein product has significant similarity to a protein involved in stabilization of plasmid pRmeGR4a of *Sinorhizobium meliloti*, which is homologous to eukaryotic cytoskeletal proteins (25). The preliminary results of our analysis suggest that the stabilizing replicating *repABC* module of paracoccal plasmids may be more complex than analogous replicons found in members of the *Rhizobiaceae*. We have initiated studies aimed at unraveling the genetic structure of the regions adjacent to the *repABC* replicon of plasmid pTAV1.

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