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Partitioning of Broad-Host-Range Plasmid RP4 Is a Complex System Involving Site-Specific Recombination†

MARTIN GERLITZ, OTTO HRABAK,‡ AND HELMUT SCHWAB*

Institut für Biotechnologie, Arbeitsgruppe Genetik, Technische Universität Graz, Schlögelgasse 9, A-8010 Graz, Austria

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The broad-host-range plasmid RP4 encodes a highly efficient partitioning system (*par*) that was previously mapped within the 6.2-kb *Pst*I C fragment. The essential functions were assigned to a region of 2.2 kb between *fiwA* and IS*21* (IS*8*). On the basis of the nucleotide sequence data of the entire *par* locus and of in vitro and in vivo expression studies, three distinct loci encoding polypeptides of 9, 18, and 24 kDa were identified. Evidence for the expression of another polypeptide was found. A putative divergent promoter was localized in an intergenic region and is suggested to be responsible for transcription of these genes. It was found that the RP4 *par* region includes a function resolving plasmid dimers. The 24-kDa polypeptide is considered to function as a resolvase, since its predicted amino acid sequence shows homology to sequences of resolvases of the Tn*3* family. Furthermore, palindromes present in the intergenic region containing the divergent promoter resemble repeat structures specific for *res* sites of Tn*3*-related transposons. However, it was found that dimer resolution itself was not sufficient for stabilization; additional functions, including the other two polypeptides, seemed to play an important role. These results suggested that RP4 contains a complex stabilization system involving resolution of plasmid dimers during cell division, thus ensuring the delivery of at least one copy to each daughter cell.

Naturally occurring plasmids are usually stably inherited under nonselective conditions. A random distribution process would be sufficient only for plasmids maintained at a high copy number. Plasmids that are present in only a few copies per cell obviously inherit functions for active partitioning systems. Such loci, termed *par* (partitioning) or *stb* (stability), have been identified in several bacterial plasmids, including F (25), R1 (15), NR1 (42), and pTAR (13), and in bacteriophage P1 (1). These stability functions apparently ensure the correct distribution of plasmid molecules to daughter cells during cell division. In each case, *cis*-acting DNA regions and *trans*-acting plasmid-encoded protein factors are involved. Participation of host factors in partitioning has also been reported (5). However, little is known regarding the molecular modes of action of different *par* systems. A recent model suggests pairing of plasmid molecules from a free pool promoted by plasmid-encoded proteins that bind to specific sites of the partitioning system (3).

Additional functions preventing plasmid-free segregants from surviving have been identified for plasmids F (19), R1 (14), and R100 (44). In all cases, plasmid-free cells generated during cell division are selectively killed. Site-specific recombination has been shown to be involved in the maintenance of P1 (4) as well as multicopy plasmids (16, 40).

The broad-host-range IncP1 plasmid RP4 and its homologs RK2, RP1, R68, and R18 have been extensively studied with respect to replication and maintenance properties (43). RP4 has a copy number of about four to seven (9) and is maintained extremely stable under nonselective conditions. By investigating a set of in vivo-generated deletion derivatives (36), a region involved in stable inheritance (*par*) has been identified. The *Pst*I C fragment of RP4 has been found to contain the complete information for an efficient system preventing the formation of plasmid-free segregants. Molecular cloning of this fragment into different replicons such as pBR322 or pACYC177 results in an extremely high degree of stabilization. Loss of plasmids cannot be detected for such clones even after more than 200 generations of growth under nonselective conditions. The stabilization effect of the *Pst*I C fragment is independent of orientation. In addition to functioning in *Escherichia coli* hosts, the stabilizing region of RP4 has been shown to function in a variety of gram-negative bacteria (35).

In this study, we present a more detailed molecular analysis of the *par* region of RP4. The essential region involved in the stabilization process was localized, and the complete nucleotide sequence of this region was determined. Several gene products encoded in this section have also been identified. One of these gene products was found to be involved in the resolution of plasmid dimers. This resolution system seems to be part of a more complex function ensuring the extremely stable inheritance of plasmid RP4.

MATERIALS AND METHODS

Strains, phages, and plasmids. *E. coli* C600 (*rec*$^+$; ATCC 33525), HB101 (*recA*; ATCC 33649), SF8 (*recBC*; R. Eichenlaub, University of Bochum, Federal Republic of Germany), and XL1 (F′ *proAB lacI*^{q}ZΔM15 Tn*10*; Stratagene, La Jolla, Calif.) were used as hosts for plasmids. Phages M13mp18 and M13mp19 and recombinants were propagated in *E. coli* JM101 (ATCC 33867). Plasmids pT7-5 and pT7-6, which differ from plasmid pT7-1 (41) in orientation of the β-lactamase gene and orientation of the multipurpose cloning site, respectively, as well as the M13mp8 derivative mGP1-2, containing the structural gene of the T7 RNA polymerase under the control of the *lac* promoter, were kindly provided

* Corresponding author.

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‡ Present address: btF-Biotechnologische Forschungs Gesellschaft, A-4021 Linz, Austria.

by Stan Tabor (Harvard University, Cambridge, Mass.). Other plasmids used were pBR322 (ATCC 37017) and pTUG3, which consists of pBR322 containing the entire *Pst*I C fragment of RP4 (35).

Media and growth conditions. *E. coli* strains were routinely grown in YT medium (10 g of tryptone [Difco Laboratories], 5 g of yeast extract [Difco], and 5 g of NaCl per liter) at 37°C. For JM101, properly supplemented M9 mineral media (24) were used when necessary. M13 recombinants were selected on 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-Gal) plates (23). Ampicillin (100 mg/liter) or tetracycline (10 mg/liter) was added for the selection of plasmids.

Plasmid stability assay. *E. coli* C600 cells containing the plasmids to be tested were grown overnight at 37°C in 50 ml of YT medium supplemented with the appropriate antibiotic (300-ml shaker flasks); 0.05 ml of this culture was transferred to 50 ml of fresh antibiotic-free YT medium (10^3-fold dilution). After incubation for 8 h at 37°C, the culture was diluted in the same manner into fresh antibiotic-free medium and grown again for 8 h at 37°C. This cycle, which gives almost exactly 10 generations, was repeated until at least 200 generations of growth under nonselective conditions were reached. After usually every 50 generations, appropriate dilutions of the cultures were plated onto nonselective YT agar plates and incubated overnight at 37°C. Approximately 200 colonies were examined for resistance pattern by replica plating, and the percentage of plasmid-free cells was determined from the ratio of sensitive to resistant colonies. Resistant and sensitive colonies were occasionally examined for the presence of plasmid DNA by the alkaline lysis method. In all cases, the results of these control experiments were consistent with the resistance pattern data.

DNA techniques. Plasmids were constructed according to standard molecular cloning techniques (23); *Bal*31 (Boehringer GmbH, Mannheim, Federal Republic of Germany) was used for the generation of nested deletions within the RP4 *par* region. After *Bal*31 nuclease digestion, the DNA ends were filled to make blunt ends with the Klenow fragment of *E. coli* DNA polymerase I and phosphorylated with T4 polynucleotide kinase. Nonphosphorylated *Bam*HI linkers (8-mer) were introduced at the deletion endpoints as described previously (37).

DNA was sequenced by the dideoxynucleotide chain termination method developed by Sanger et al. (34), using M13-generated single-stranded DNA as templates. Reagent kits employing either the Klenow fragment of *E. coli* DNA polymerase I or reverse transcriptase (Bio-Rad, Richmond, Calif.), or Sequenase (United States Biochemicals, Cleveland, Ohio) or *Taq* polymerase (Promega Biotec), were used as instructed by the suppliers. 7-Deaza-dGTP or dITP was used occasionally to reduce secondary-structure formation. The programs of the Gene Master system (Bio-Rad) were used to handle and analyze sequence data.

Identification of polypeptides. For in vitro transcription and translation, a commercial system (Zubay system from Amersham International, Amersham, United Kingdom) based on S30 lysates of *E. coli* was used. Highly purified supercoiled plasmid DNA or linear DNA fragments were applied as templates. The T7 RNA polymerase-based in vivo system was used as described previously (41) except that strain XL1 was used as the host. Specific transcription from the T7 promoter was induced by infection with phage mGP1-2, which contains the T7 RNA polymerase gene under the control of the *E. coli lac* promoter, in the presence of isopropyl-β-D-galactopyranoside (IPTG). Synthesized polypeptides were labeled with [^{35}S]methionine and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography.

Nucleotide sequence accession number. The sequence reported has been deposited in the GenBank data library under accession number M37620.

RESULTS

The *par* locus of RP4 comprises a 2.2-kb region between IS*21* (IS*8*) and *fiwA*. We previously reported (35) that the *Pst*I C fragment of RP4 contains the genetic information for a very efficient stabilization process. To locate gene functions involved in the stabilization process more precisely, various deletion derivatives and subclones of the *Pst*I C fragment were constructed from pTUG3, using known restriction sites and pBR322 as the vector. In addition, progressive deletions starting from the single *Hpa*I site in pOH24 (Fig. 1), which was shown to be located outside the essential region for the partitioning function, were generated by nuclease *Bal*31 digestion. *Bam*HI linkers were introduced at the deletion endpoints, and a set of derivatives with deletions extending from the *Hpa*I site into the *par* region was selected (pGMA6 to pGMA28). A second set of derivatives with progressive deletions into both directions from the *Sal*I site was constructed from pGMA27 by using a coincident strategy (pGMA41 to pGMA49). Schematic maps of the relevant derivatives are summarized in Fig. 1. The exact deletion endpoints of *Bal*31-generated derivatives are indicated in the nucleotide sequence shown in Fig. 4.

Plasmid stability under nonselective conditions was determined for all derivatives, using *E. coli* C600 as the host strain. Some of the plasmids were also tested by using the recombination-deficient host *E. coli* HB101, but no significant differences were observed. The plasmids could be combined into four groups characterized by different kinetics of plasmid loss (Fig. 2). Group a plasmids expressed the same high stability as did plasmids containing the entire *Pst*I C fragment (e.g., pTUG3). No plasmid-free cells were detected under the conditions of the stability assay. All of these plasmids contain the region between the *Sph*I site at position 33.17 kb of the RP4 map (22) and the deletion endpoint of pGMA27, the smallest fully stabilized derivative found.

A stabilization effect was also seen with group b plasmids. However, in contrast to the group a plasmids, a certain percentage of plasmid-free cells was reproducibly detected during prolonged growth under nonselective conditions (5 to 10% after 200 generations). All of these group b plasmids contain the region to the right of the *Sal*I site (Fig. 1), but parts of the region left of this *Sal*I site, at least the small 0.48-kb *Sph*I fragment (Fig. 1), are deleted.

When analyzing the *Bal*31-generated derivatives with deletions extending from the right side into the *par* region, it was found that pGMA27 and all derivatives having smaller deletions (e.g., pGMA28) were fully stabilized. Although derivatives pGMA23 and pGMA25 were repeatedly examined, they could not be clearly classified. In most cases, these plasmids showed no stabilization effect. However, in about half of the experiments a partial stabilization effect, as was found with group b plasmids, could be observed. All derivatives having larger deletions (e.g., pGMA21) clearly showed no stabilization effect, and the kinetics of plasmid loss were comparable to those of pBR322 (group c). Deletions in both directions from the *Sal*I site usually resulted in loss of any stabilizing capability (group c). In some cases, a destabilization effect was observed (group d). Only plasmid pGMA41 showed a partial stabilization effect and was classified as a group b plasmid.

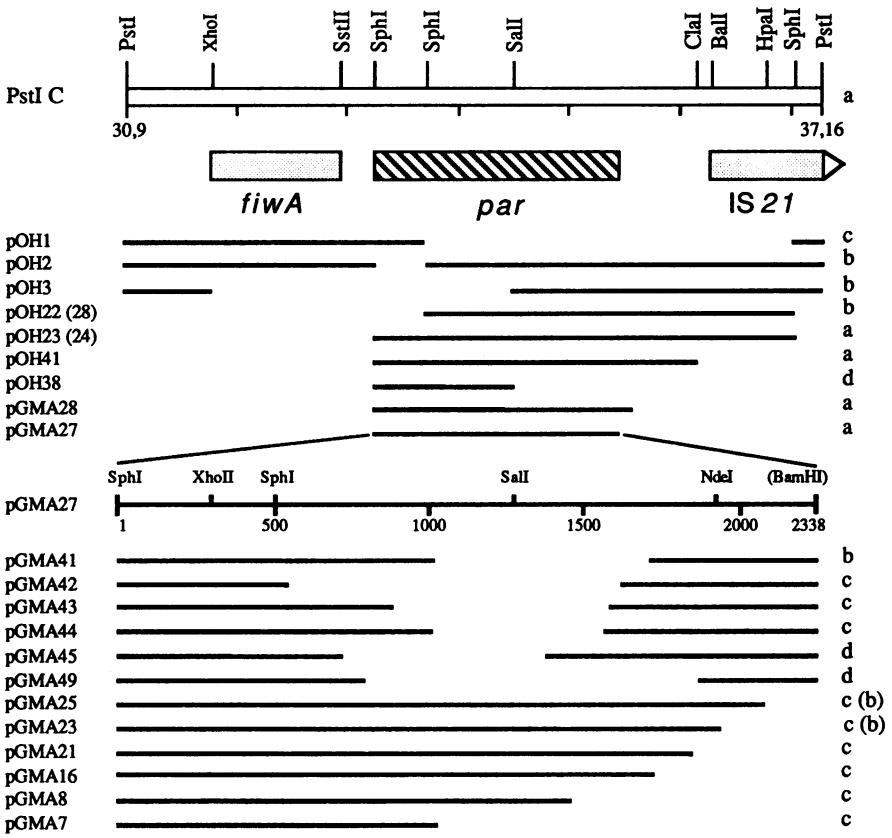


FIG. 1. Deletion analysis of the *Pst*I C fragment of plasmid RP4. pOH plasmids were constructed by deleting fragments from pTUG3 or by recloning respective fragments of the *Pst*I C fragment into pBR322, using the known restriction sites (22). Fragments in pOH1, pOH2, and pOH3 are cloned into the *Pst*I site of pBR322, and fragments in pOH22, pOH23, and pOH24 are cloned into the *Sph*I site. pOH38 and pOH41 are deletion derivatives of pOH24 and pOH23, respectively. Numbers in parentheses indicate plasmids with the fragment in the inverse orientation. The pGMA plasmids are *Bal*31-generated deletion derivatives. Plasmids pGMA7 to pGMA28 were constructed from pOH24. The deletions in these derivatives start from the single *Hpa*I site and extend from IS*21* into the *par* region and into the tetracycline resistance gene of pBR322, respectively. Plasmids pGMA41 to pGMA49 have deletions extending from the *Sal*I site into both sides of the *par* region and were constructed from pGMA27. All *Bal*31-generated derivatives contain an additional *Bam*HI site at the deletion junction. For better presentation of smaller derivatives, the scale for pGMA27 was enlarged. Stability of the plasmids in *E. coli* C600 is indicated on the right. The classification into groups a to d corresponds to the plasmid loss kinetics shown in Fig. 2. The scale (kilobase coordinates) and restriction sites correspond to the physical map of RP4 (22). The patterned bars beneath the open bar representing the *Pst*I C fragment show the locations of the partitioning system (*par*) and adjacent functions of RP4, *fiwA* (11), and IS*21* (IS*8*) (6, 31).

On the basis of these data, we assigned the RP4 *par* locus to a region of about 2.2 kb between the *Sph*I site at position 33.17 kb and the deletion endpoint of pGMA27 at position 35.4 kb of the RP4 restriction map (22). Small deletions on either side of this 2.2-kb fragment reduced or completely abolished stabilizing capability.

Complete nucleotide sequence of the RP4 *par* region. The complete nucleotide sequence of the RP4 *par* fragment present in pGMA28 (Fig. 1) was determined. Appropriate DNA fragments from the *Pst*I C fragment or from *Bal*31-generated deletion derivatives were subcloned into M13mp18 or M13mp19 and sequenced by the dideoxy-chain termination method. The sequencing strategy is outlined in Fig. 3. Problems due to marked tendencies of some regions to form secondary structures could be overcome by using different DNA polymerases and nucleotide analogs. The complete nucleotide sequence of the RP4 fragment present in pGMA28 is shown in Fig. 4.

A strong binding site for *E. coli* RNA polymerase has been described as being present at RP4 map position 35 kb (28), corresponding to nucleotide positions 1800 to 1900. Using a computer program based on data for 263 *E. coli* promoters (17), sequences fitting well to the σ^{70} promoter consensus were identified on both strands within this region (indicated in Fig. 4). This finding provides evidence for the presence of a divergent promoter system in this region which mediates transcription in both directions. The sequences of these putative promoter regions do not contain inverted sequence repetitions specific for *korB*-regulated genes involved in plasmid maintenance (29). In some of the identified putative promoter sequences, a motif at position −12 (tGCt), specific for promoters functioning well in *Pseudomonas* spp. (7) and classified as group A54 (8), could be found (indicated in Fig. 4). This class represents a set of regulated promoters transcribed by the *ntrA* (σ^{57})-like RNA polymerase holoenzyme. This motif is also present in the promoters of the RP4 genes *trfA*, *trfB*, *kilA*, and *kilB* (29).

The entire sequence is characterized by an outstanding accumulation of both direct and inverted repeats. The most significant structures are indicated in Fig. 4. However, at this stage of our studies there is no definitive evidence for a biological function of such structures. The inverted repeat between nucleotides 2290 and 2307 would give rise to a G+C-rich stem-loop, followed by an A+U-rich stretch that

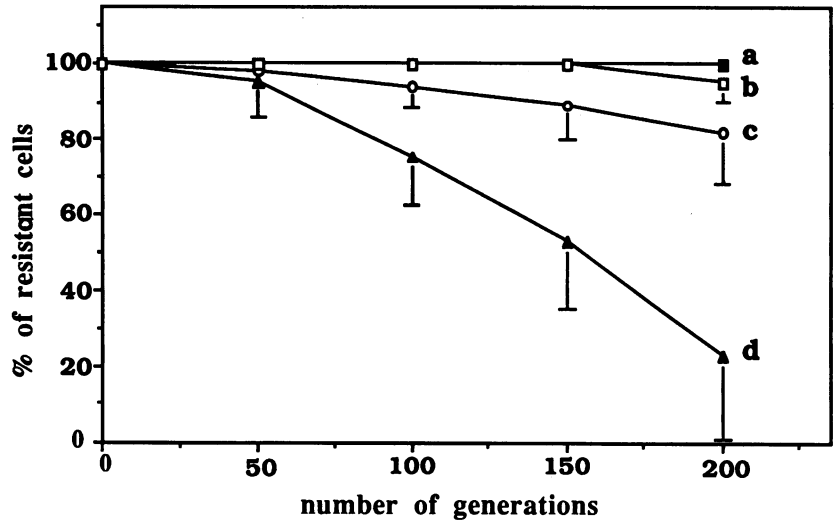


FIG. 2. Kinetics of the loss of plasmids containing different parts of the RP4 *par* region. All plasmids were tested in an *E. coli* C600 host. Cells grown overnight at 37°C in selective medium were repeatedly subcultured by 1,000-fold dilution into nonselective medium, followed by 8 h of incubation; this procedure yielded almost exactly 10 generations per cycle. Appropriate dilutions were plated onto antibiotic-free YT plates, and at least 200 colonies were checked for resistance pattern by replica plating to determine the percentage of plasmid-free cells. The letters a to d refer to groups of plasmids showing similar behavior: a, pTUG3, pOH23, pOH24, pOH41, pGMA27, and pGMA28; b, pOH2, pOH3, pOH22, pOH28, and pGMA41; c, pBR322, pOH1, pGMA7 to pGMA21, pGMA23, pGMA25 (in two of four experiments, plasmids pGMA23 and pGMA25 showed loss kinetics comparable to those of group b), pGMA42, and pGMA44; d, pOH38, pGMA43, pGMA45, and pGMA49. Curves represent the values obtained with the most stable plasmid of a group. All other plasmids of a group showed values ranging within the borders indicated. The data represent mean values of two parallel determinations of at least two independent experiments.

resembles a Rho-independent transcription termination site (30). Two large palindromes, each characterized by one mismatch, as well as a smaller perfect palindrome are present in the intergenic region containing the divergent promoters. In addition, this region contains various smaller direct and inverted repeats. A further region characterized by a significant accumulation of repeat structures is located between bases 1100 and 1350.

Several open reading frames preceded by putative ribosome-binding sites can be found on both strands (see Fig. 6). These features are discussed below, together with data on identified polypeptides encoded in the *par* region of RP4.

Detection of polypeptides encoded by the RP4 *par* region. Experiments to identify *par* polypeptides by in vitro transcription-translation in a Zubay system gave evidence for expression of a 9-kDa protein from plasmids containing the entire *par* region or the region to the right of the *Sal*I site (Fig. 5A). A polypeptide of the same size was also expressed in an in vivo T7 RNA polymerase-promoter system when the direction of transcription was from the *Sph*I site to the *Bam*HI site in pGMA28 (Fig. 5B). When the subfragment of pGMA49 from bases 1874 to 2241 was analyzed with this system (data not shown), a 9-kDa protein was again found. Thus, this protein should be the product of open reading

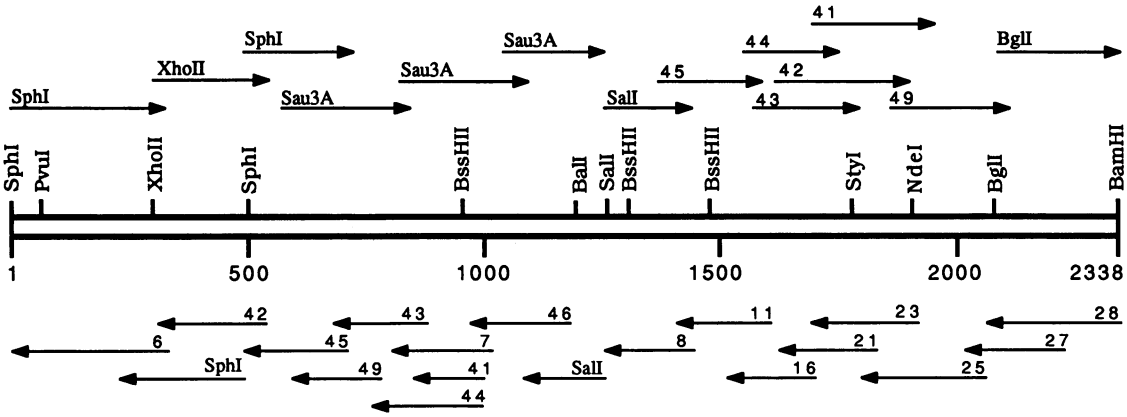
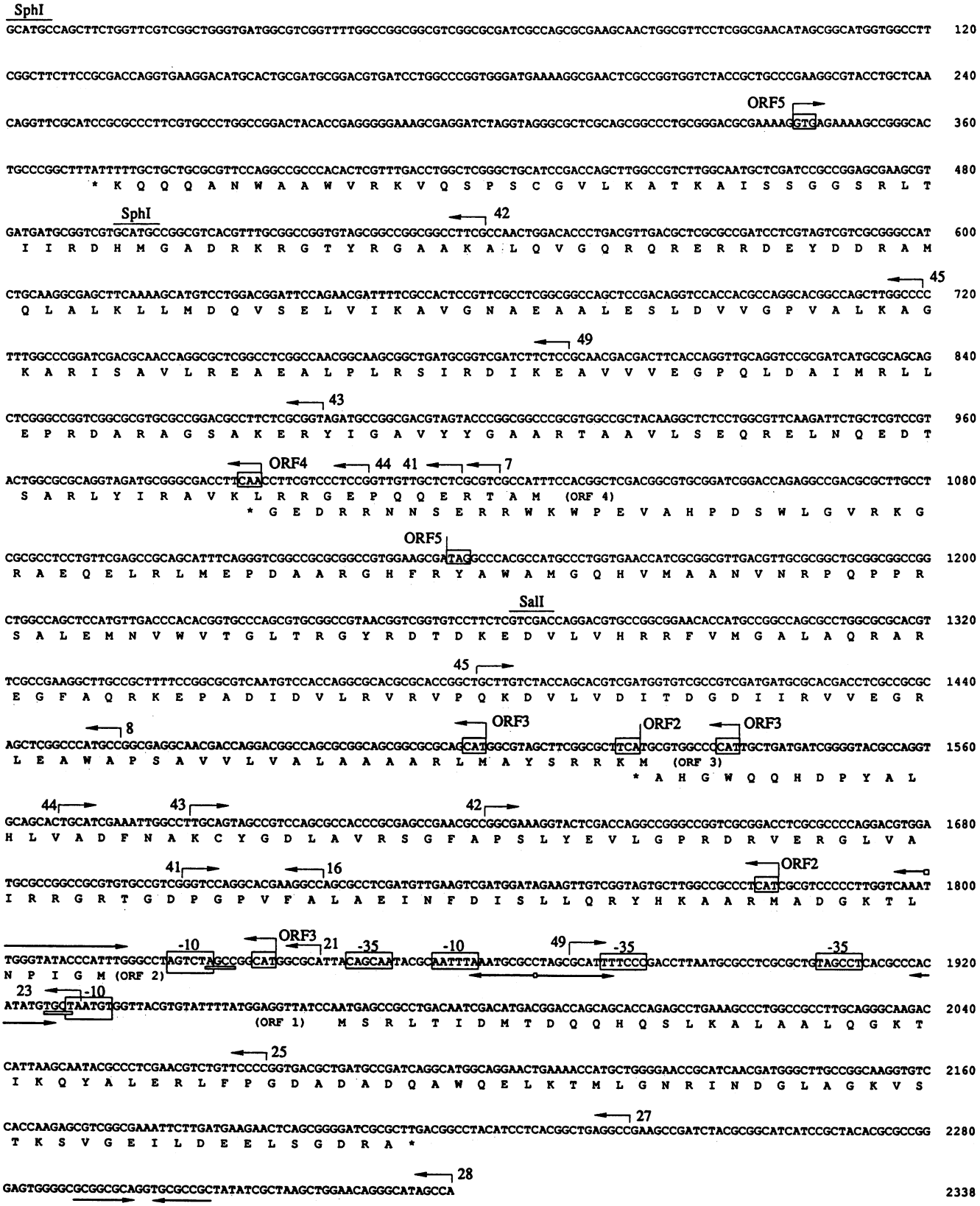


FIG. 3. Sequencing strategy. The central bar represents the region of RP4 present in pGMA28. Sequencing was based on the set of the ordered *Bal*31-generated deletions. Proper fragments were cloned into M13mp18 and M13mp19 by using the *Bam*HI site introduced at the deletion junction. Furthermore, specific fragments of pGMA28 were directly cloned into M13mp18 and M13mp19 by using already known restriction sites. Numbers refer to the respective pGMA clones; for directly cloned subfragments, the relevant restriction sites used are indicated. The direction of sequencing is marked by arrows. The length of each arrowed bar corresponds to the number of bases that could be read from the gels.


SphI
ORF5
SphI
42
45
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ORF4
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ORF5
SalI
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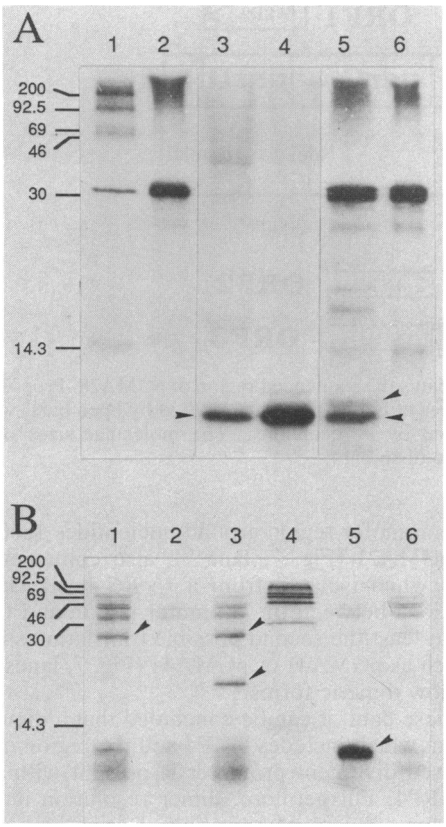

FIG. 5. Polypeptides encoded by the RP4 *par* region. (A) Proteins synthesized in a DNA-dependent in vitro transcription-translation system (Amersham). Highly purified DNA (2 to 5 μg) was applied to the system, and polypeptides were synthesized by incubation for 40 min at 37°C in the presence of 20 μCi of [^{35}S]methionine. After denaturation, proteins were separated by electrophoresis on a 20% sodium dodecyl sulfate-polyacrylamide gel and visualized by fluorography. Lanes: 1, standard; 2, pAT153 (control DNA supplied with the system); 3, linear *Pst*I C fragment; 4, pOH41; 5, pOH22; 6, pOH38. Lanes 3, 5, and 6 contained supercoiled plasmid DNA. (B) In vivo analysis by a strand-specific T7 RNA polymerase-promoter system. Fragments of the RP4 *par* region were cloned into pT7-5 or pT7-6. The resulting plasmids were transformed into *E. coli* XL1. Characterized transformants were grown in YT medium to an optical density at 690 nm of 0.5 and then infected with phage mGP1-2. After induction of T7 RNA polymerase with IPTG (1 mM), polypeptides were labeled by 10-min incubation at 37°C in the presence of 10 μCi of [^{35}S]methionine and 0.2 mg of rifampin per ml. After separation on a higher cross-linked 15% sodium dodecyl sulfate-polyacrylamide gel (10:1 acrylamide/bisacrylamide), proteins were visualized by fluorography. Lanes: 1, *Sph*I-*Sal*I fragment of pOH38 in pT7-6; 2, pT7-5; 3, entire *Bam*HI-*Sph*I *par* fragment of pGMA28 cloned in pT7-5; 4, standard; 5, same fragment as in lane 3 cloned in pT7-6; 6, pT7-6. Sizes (in kilodaltons) of proteins of the [^{14}C]-methylated protein standard mix are shown on the left. Bands representing Par proteins are marked by arrowheads.

frame 1 (ORF1) (Fig. 6). The second ATG at position 1964 is the most probable start codon, since the size of the resulting possible coding region corresponds to the apparent mass of the protein. Also, there is a remarkable putative ribosome-binding site: 13 of 15 bases are homologous to the 3′ end of the 16S rRNA. A second polypeptide of about 10 kDa is expressed in the Zubay system from clones missing the region left of the *Sal*I site, such as pOH22 (Fig. 5A, lane 5) or pOH3 (data not shown). With the T7 system, it was not possible to clearly detect a corresponding polypeptide encoded by the pGMA28 *par* fragment. Unfortunately, background bands probably corresponding to vector-encoded products were seen at the respective positions on the autoradiograph when the vector itself was analyzed (Fig. 5B, lane 2). ORF2 (Fig. 6), which has two possible translation start sites (ATG at positions 1780 and 1813) preceded by possible ribosome-binding sites, would fit quite well to encode a 10-kDa polypeptide. Since with pOH41 and with the *Pst*I C fragment the 10-kDa protein was not detected in the Zubay system, a regulated expression for this polypeptide that depends on genetic information present in the region left of the *Sal*I site might be postulated. However, further work is necessary to clarify the situation with ORF2.

It was not possible to clearly detect additional polypeptides specific for the *par* region with the Zubay system. Two additional polypeptides, of about 18 and 24 kDa, could be definitely detected with the T7 system when transcription in the analyzed *par* fragment of pGMA28 was in the direction of the *Sph*I site (Fig. 5B, lane 3). The 24-kDa polypeptide was also expressed from a subfragment containing only the region to the left of the *Sal*I site (Fig. 5B, lane 1). This polypeptide should therefore be encoded by ORF4. Two possible translation start sites (ATG at position 1029; TTG at position 993) giving rise to putative polypeptides of about 23 and 24 kDa, respectively, were found. The TTG is preceded by a more reliable ribosome-binding site. Since a double band was seen on most gels (Fig. 5B, lanes 1 and 3), it is also possible that both translation start sites can be used, at least in the T7 promoter-polymerase system. The 18-kDa protein may be encoded by ORF3 starting at position 1502 (ATG) or at position 1523 (ATG), giving rise to putative polypeptides of about 19 and 20 kDa. Another possible start codon situated nearby at position 1535 as well as an additional ATG far upstream at position 1835 are not preceded by any reliable ribosome-binding site. Since there is good evidence that the 9-, 10-, 18-, and 24-kDa proteins are encoded by ORF1, ORF2, ORF3, and ORF4, we designated genetic loci for the proposed coding regions (see Fig. 4) as indicated in Fig. 6.

It should be noted that the stop codons of ORF2 and ORF3 overlap the predicted start codons of ORF3 and ORF4, respectively. Interestingly, the same situation was found with the open reading frames of the *istA* and *istB* genes of IS*21* (31), which are located adjacent to the *par* region.

Another interesting finding is that ORF5 completely cov-

FIG. 4. Nucleotide sequence of the 2.3-kb *par* region of plasmid RP4 present in pGMA28. The deletion endpoints of relevant *Bal*31-generated deletion derivatives are indicated by angled arrows. The numbers next to the arrows refer to the corresponding pGMA plasmids. Significant repeat structures are indicated by arrows below the sequence; mismatches are indicated by small boxes within these arrows. The deduced amino acid sequences for the polypeptides that could be adjoined to open reading frames are written in the one-letter code below the nucleotide sequence. The deduced amino acid sequences are given from the first possible start codon preceded by a reliable ribosome-binding site. Further putative start codons within these frames and of the open reading frames for which no polypeptides could definitively be assigned are marked by boxes with an angled arrow indicating the direction of translation. Stop codons are marked by boxes with a vertical bar. The −10 and −35 regions of the promoter consensus sequences found by computer analysis in the intergenic region between ORF1 and ORF2 are boxed. The tGCt motif of *Pseudomonas* promoters (7, 8) is marked by a open bar below the sequence.

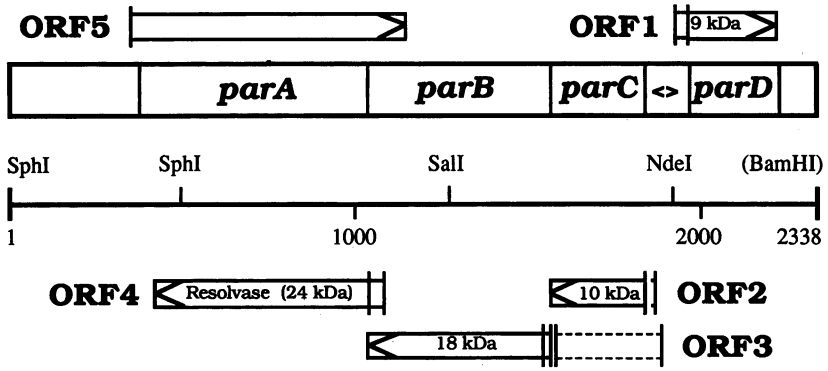

FIG. 6. Relevant open reading frames in the RP4 *par* region. The central bar indicates the sequenced region of pGMA28. Proposed genetic loci are indicated within. < >, The divergent promoter. Relevant open reading frames of both strands are marked by open bars, with arrows inside indicating the direction of transcription. Possible start codons are marked by vertical lines. The molecular sizes of detected polypeptides that could be assigned to open reading frames are indicated within the open bars.

ers the region of ORF4 on the complementary strand (Fig. 6). A possible translation start site for ORF5 would be a GTG at position 343. Until now, no indication for the expression of a polypeptide from ORF5 has been found.

Identification of a dimer resolution system. All of the open reading frames discussed above were compared by dot matrix analysis at the nucleotide and amino acid sequence levels with all published sequences of the *par* systems of plasmids F, R1, pTAR, and P1, including the *ccd* and *hok/sok* systems of F and R1 and the related *pem* system of R100. However, no significant similarities were detected. From routine plasmid isolations, we got the impression that resolution of dimers might be involved in the stabilization process mediated by the RP4 *par* region (O. Hrabak, Ph.D. dissertation, Technical University of Graz, Graz, Austria, 1987). It was possible to demonstrate a specific phenotypic effect attributed to expression of ORF4 (Fig. 7). Isolated plasmid DNA of pBR322 derivatives containing different parts of the *par* region consists of only monomeric molecules when a functional ORF4 including the promoter region is present. Plasmid DNA from derivatives either deleted in parts of ORF4, such as pOH22 (Fig. 7, lane 1), or lacking the proposed promoter region around nucleotides 1800 to 1900, such as pGMA21 (Fig. 7, lane 7), also contained dimeric molecules when isolated from a *recBC* host. Derivatives with deletions between the promoter region and ORF4 but retaining at least the second possible translation start site of ORF4, such as pGMA41 or pGMA44 (Fig. 7, lanes 2 and 3), did not show dimeric forms.

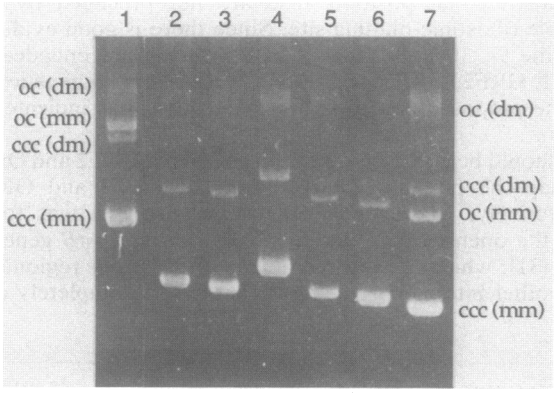

FIG. 7. Effect of resolvase on plasmid DNA. pBR322 derivatives containing different parts of the RP4 *par* region were transformed into *E. coli* SF8. Plasmid DNA was extracted from the respective transformants by the alkaline lysis method (minipreparation) and analyzed on a 0.7% agarose gel. Lanes: 1, pOH22; 2, pGMA44; 3, pGMA41; 4, pGMA27; 5, pGMA25; 6, pGMA23; 7, pGMA21. Abbreviations: mm, monomer; dm, dimer; ccc, covalently closed circle; oc, open circle.

From these data, it can be concluded that a dimer resolution system which includes ORF4 and the region containing the proposed divergent promoter is present within the *par* region of RP4. Furthermore, dimer resolution itself is not sufficient for efficient stabilization. Derivatives pGMA41, pGMA44, pGMA23, and pGMA25 express resolution activity but show either no or only a reduced stabilizing effect (Fig. 2).

Comparison of amino acid sequences revealed a pronounced homology of the deduced polypeptide of ORF4 to resolvases of transposons of the Tn*3* family (Fig. 8), being highest to that of Tn*1721* (33). The conserved regions, including the presumptive serine involved in the 5'-phosphoseryl linkage to DNA and the *cro*-like DNA-binding domain at the C terminus (38), are all present in the deduced sequence of the ParA protein. From these data, it can be concluded that ORF4 encodes a resolvase. The region between ORF1 and ORF2 including the three palindromes (Fig. 4) may represent a possible *cis*-acting site for resolvase action. The sequence features of this region resemble those of *res* sites of known site-specific resolution systems (18, 38).

DISCUSSION

Our previously reported studies (35) demonstrated that the broad-host-range plasmid RP4 contains a highly efficient stabilization system that is located within the 6.2-kb *Pst*I C fragment. In this study, it was shown that all essential functions are located on a 2.2-kb region between coordinates 33.17 and 35.4 kb of the RP4 map (22). A complex system consisting of at least three polypeptides transcribed from a divergent promoter (summarized in Fig. 6) and a putative *cis*-acting site was found to be responsible for the extremely high segregational stability of the plasmid RP4. Comparison of sequences with essential components of other partitioning systems revealed no significant similarity. Therefore, it can be assumed that the RP4 system may represent a novel system.

Deletions extending from IS*21* (IS*8*) into the *par* region have no effect on stability as long as ORF1 remains intact

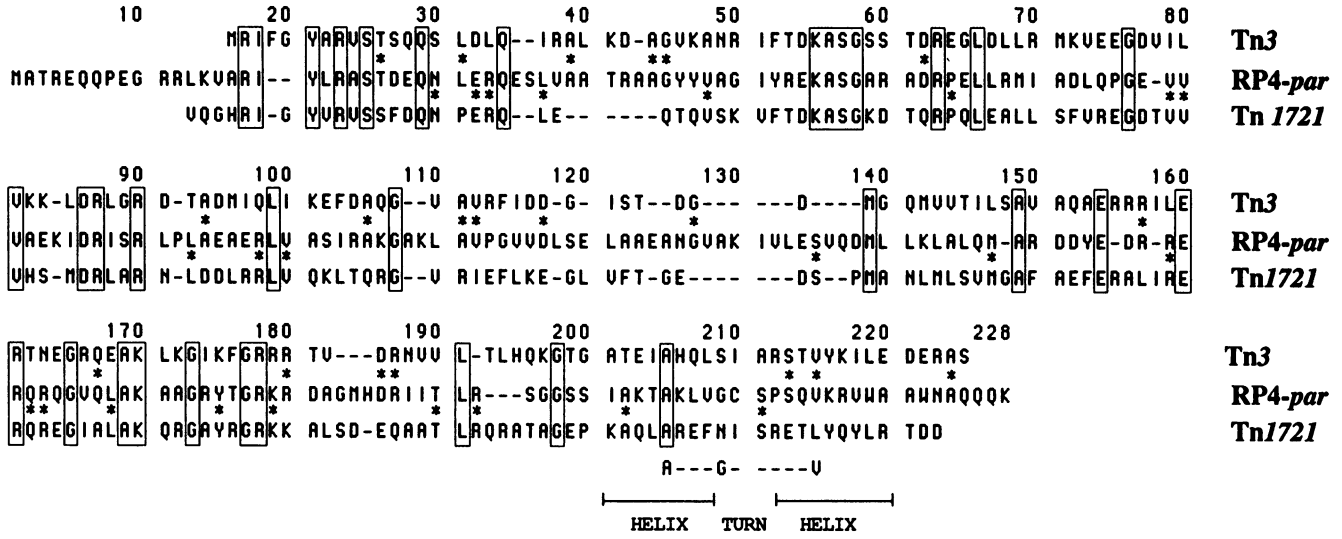

FIG. 8. Alignment of the amino acid sequences of the resolvases of the RP4 partitioning system (*parA*), Tn*3*, and Tn*1721*. Amino acids highly conserved within the sequences of the whole Tn*3* resolvase family (38) are boxed. Additional homologies of the RP4 ParA protein to the resolvase protein of Tn*3* or Tn*1721* are marked by asterisks. The potential helix-turn-helix DNA-binding motif (27) at the carboxyl-terminal end of the resolvases is indicated below; its highly conserved amino acids are shown separately.

(pGMA27). Deletion of ORF1 or parts thereof (pGMA23 and pGMA25) clearly affects the stabilizing property. The finding of a partial stabilization effect in some experiments with plasmids pGMA23 and pGMA25 may indicate a role of the ORF1 protein in compensating environmental or physiological conditions. Because of slight experimental variations during different stability assays, cell populations might have been exposed to changes of environmental parameters (e.g., substrate limitations or oxygen supply), which in turn may have resulted in varying influences on the maintenance of those plasmids lacking an intact *parD* region. Roberts et al. (32) also analyzed various deletion derivatives of the RP4 *par* region. In contrast to our studies based on multicopy replicons (pBR322), low-copy replicons were used in their studies. However, the results obtained with the two systems are in general comparable. Roberts et al. also found that derivatives having deletions extending into the region containing ORF1 were characterized by a drastic decrease but not by a complete loss of stabilizing capability. It is notable that the 9-kDa protein encoded by ORF1 is very efficiently expressed in the in vitro Zubay system as well as in the in vivo T7 polymerase-promoter system. Therefore, we assume that a sufficient level of ORF1 protein might be needed to ensure effective plasmid maintenance. Since nothing is known about in vivo expression levels of the ORF1 protein under natural conditions of plasmid maintenance, further studies are necessary.

We demonstrated that the RP4 *par* region contains a function which prevents the appearance of plasmid dimers. It has also been reported that the *Pst*I C fragment contains a multimer resolution function (N. J. Grinter and P. T. Barth, Abstr. Conf. Promiscuous Plasmids of Gram-Negative Bacteria, 1987). Roberts et al. (32) were able to define *cis*- and *trans*-acting regions for this multimer resolution system and located both regions within the boundaries of the partitioning system. We found that the region between ORF1 and ORF2, which contains the putative divergent promoter and the set of the three palindromes (Fig. 4), is essential for the stabilization process. No stabilizing effect could be seen when this region or parts thereof were deleted. This region shows a pronounced similarity in organization to the *res* site of Tn*3* (18, 38) and could likely be regarded as the *cis*-acting site for the resolution system. In fact, it was demonstrated that this region represents the *cis*-acting part of the site-specific recombination system included in the RP4 *par* region. Furthermore, it was found that site-specific recombination is independent of site orientation, indicating the involvement of a recombinase belonging to a class that can act on both direct and inverted repeats (32). We found that the amino acid sequence of the resolvase of the RP4 partitioning system (*parA*) has highest homology to the resolvase of transposon Tn*1721*, which was shown to belong to such a class of recombinases (2).

An interesting similarity was found between the loci of the *par* resolvase of RP4 and the D protein of the mini-F plasmid. Both proteins have resolvase activity but share no significant sequence homologies. However, the situation that the complete reading frame of the resolvase (ORF4) is mirrored in the opposite strand by an open reading frame (ORF5) has also been found with the D protein of mini-F (21). Whether this fact has any significance cannot be decided at this stage of investigation.

Partial or complete removal of the structural gene of the resolvase (*parA*) and retention of the proposed resolution site as well as *parD* (e.g., pOH3 and pOH22) results in that phenotype of stabilization at a reduced level (group b plasmids; Fig. 2). An explanation for this effect would be that a resolvase activity provided by the host can partially complement the plasmid-encoded resolvase of the partitioning system. Involvement of host factors in plasmid site-specific recombination has been shown for multicopy plasmids. For example, at least two unlinked functions encoded on the *E. coli* chromosome can act on the *cer* site of plasmid ColE1 (39).

There is clear evidence that a polypeptide can be expressed from *parB*. Derivatives deleted of the *parB* region but retaining an intact resolution system and *parD* are unstable (pGMA44) or at most partially stabilized (pGMA41). Therefore, we assume that *parB* is an essential part of the RP4 partitioning system.

Complementation and incompatibility studies have been performed with different parts of the RP4 *Pst*I C fragment (M. Gerlitz, unpublished data). Weak incompatibility was found between pMB1 and p15A replicons containing the entire *Pst*I C fragment. When distinct parts of the *Pst*I C fragment were tested for incompatibility or complementation, the situation turned out to be rather complex, since multiple interactions, including drastic destabilization and oscillation effects, occur, indicating impairment of regulatory systems. Blocking of the function of partitioning systems, destabilization effects, and inhibition of plasmid maintenance as a result of overexpression of Par proteins have been reported for other plasmids (1, 12, 20). The effect of a higher rate of plasmid loss found with some deletion derivatives (group d plasmids; Fig. 2) could also be the result of disturbing regulatory circuits.

In conclusion, the RP4 *par* region represents a complexly organized system. The resolution of plasmid multimers does not seem to be the main principle of the stabilization process. No detectable amounts of plasmid multimers could be found in plasmid DNA preparations from *rec*$^+$ or *recA* hosts even though all plasmids that do not contain an intact RP4 *par* region are unstable in these hosts. Larger amounts of dimers with plasmids lacking an intact resolution system were observed only when a *recBC* host was used. The *recBC* gene product (exonuclease V) is known to be involved in the resolution of recombination intermediates (10). In addition, it was shown that derivatives having an intact resolution system but lacking other segments, such as *parD* (pGMA23 and pGMA25) or *parB* (pGMA44 and pGMA41), are unstable or at least not fully stabilized. We speculate that the site-specific recombination system of RP4 might be part of an efficient partitioning mechanism. It has been postulated that partitioning involves tight pairing of two plasmid molecules by DNA-protein interaction (3, 26). Furthermore, the absence of incompatibility with the NR1 system is explained by a very efficient pairing of plasmids during or immediately after replication, before they enter a free pool (3). One way of explaining the mechanism of the RP4 *par* system would include the involvement of the formation of plasmid dimer intermediates that are resolved by the site-specific resolution system during cell division, thus ensuring the exact partitioning of at least one copy to each daughter cell. This assumption is further supported by the fact that the chromosomally encoded integration host factor, an essential component of the site-specific integration-recombination system of phage λ, has been shown to be part of the protein-DNA complex formed at the *parS* site of P1 (1, 3).

Further study of the interaction of the various components of the RP4 *par* region is in progress.

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LITERATURE CITED

1. **Abeles, A. L., S. A. Friedman, and S. J. Austin.** 1985. Partition of unit-copy miniplasmids to daughter cells. III. The DNA sequence and functional organization of the P1 partition region. J. Mol. Biol. **85**:261–272.
2. **Altenbuchner, J., and R. Schmitt.** 1983. Transposon Tn*1721*: site specific recombination generates deletions and inversions. Mol. Gen. Genet. **190**:300–308.
3. **Austin, S. J.** 1988. Plasmid partition. Plasmid **20**:1–9.
4. **Austin, S., M. Ziese, and N. Sternberg.** 1981. A novel role for site-specific recombination in maintenance of bacterial replicons. Cell **25**:729–736.
5. **Davis, M. A., and S. J. Austin.** 1988. Recognition of the P1 plasmid centromere analog involves binding of the ParB protein and is modified by a specific host factor. EMBO J. **7**:1881–1888.
6. **Depicker, A., M. de Block, D. Inze, M. van Montagu, and J. Schell.** 1980. IS-like element IS*8* in RP4 plasmid and its involvement in cointegration. Gene **10**:329–338.
7. **Deretic, V., J. F. Gill, and A. M. Chakrabarty.** 1987. Alginate biosynthesis: a model system for gene regulation and function in *Pseudomonas*. Bio/Technology **5**:469–477.
8. **Deretic, V., W. M. Konyecsni, C. D. Mohr, D. W. Martin, and N. S. Hibler.** 1989. Common denominators of promoter control in *Pseudomonas* and other bacteria. Bio/Technology **7**:1249–1254.
9. **Figurski, D. H., and D. Helinski.** 1979. Replication of an origin containing derivative of plasmid RK2 dependent on a plasmid function in *trans*. Proc. Natl. Acad. Sci. USA **76**:1648–1652.
10. **Fishel, R. A., A. A. James, and R. Kolodner.** 1981. *recA* independent general genetic recombination of plasmids. Nature (London) **294**:184–186.
11. **Fong, S. T., and V. A. Stanisich.** 1989. Location and characterization of two functions on RP1 that inhibit the fertility of the IncW plasmid R388. J. Gen. Microbiol. **135**:499–502.
12. **Funnell, B.** 1988. Mini-P1 plasmid partitioning: excess ParB protein destabilizes plasmids containing the centromere *parS*. J. Bacteriol. **170**:954–960.
13. **Gallie, D. R., and C. I. Kado.** 1987. *Agrobacterium tumefaciens* pTAR *parA* promoter region involved in autoregulation, incompatibility and plasmid partitioning. J. Mol. Biol. **193**:465–478.
14. **Gerdes, K., F. W. Bech, S. T. Jorgensen, and A. Lobner-Olesen.** 1986. Mechanism of postsegregational killing by the Hok gene product of the *parB* system of plasmid R1 and its homology with the *relF* gene product of the *E. coli relB* operon. EMBO J. **5**:2023–2029.
15. **Gerdes, K., and S. Molin.** 1986. Partitioning of plasmid R1: structural and functional analysis of the *parA* locus. J. Mol. Biol. **190**:269–279.
16. **Hakkaart, M. J. J., P. J. M. van den Elzen, E. Veltcamp, and H. J. J. Nijkamp.** 1984. Maintenance of multicopy plasmid CloDF13 in *Escherichia coli* cells: evidence for site specific recombination at *parB*. Cell **36**:203–209.
17. **Harley, C. B., and R. P. Reynolds.** 1987. Analysis of *E. coli* promoter sequences. Nucleic Acids Res. **15**:2343–2361.
18. **Hatfull, G. F., and N. D. F. Grindley.** 1989. Resolvases and DNA invertases: a family of enzymes active in site-specific recombination, p. 357–396. *In* R. Kucherlapati and G. R. Smith (ed.), Genetic recombination. American Society for Microbiology, Washington, D.C.
19. **Hiraga, S., A. Jaffe, T. Ogura, H. Mori, and H. Takahashi.** 1986. F plasmid *ccd* mechanism in *Escherichia coli*. J. Bacteriol. **166**:100–104.
20. **Kusukawa, N., H. Mori, A. Kondo, and S. Hiraga.** 1987. Partitioning of the F plasmid: overproduction of an essential protein for partition inhibits plasmid maintenance. Mol. Gen. Genet. **208**:365–372.
21. **Lane, D., R. de Feyter, M. Kennedy, S.-H. Phua, and D. Semon.** 1986. D protein of mini F plasmid acts as a repressor of transcription and as a site specific resolvase. Nucleic Acids Res. **14**:9713–9728.
22. **Lanka, E., R. Lurz, and J. P. Fürste.** 1983. Molecular cloning and mapping of *Sph*I fragments of plasmid RP4. Plasmid **10**:303–307.
23. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

24. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
25. **Mori, H., A. Kondo, A. Ohshima, T. Ogura, and S. Hiraga.** 1986. Structure and function of the F plasmid genes essential for partitioning. J. Mol. Biol. **192**:1–15.
26. **Nordström, K., and S. J. Austin.** 1989. Mechanisms that contribute to the stable segregation of plasmids. Annu. Rev. Genet. **23**:37–69.
27. **Pabo, C. O., and R. T. Sauer.** 1984. Protein-DNA recognition. Annu. Rev. Biochem. **53**:293–321.
28. **Pansegrau, W., L. Miele, R. Lurz, and E. Lanka.** 1987. Nucleotide sequence of the kanamycin resistance determinant of plasmid RP4: homology of other aminoglycoside 3′-phosphotransferases. Plasmid **18**:193–204.
29. **Pinkney, M., and C. M. Thomas.** 1987. Replication and maintenance of promiscuous plasmids of Gram-negative bacteria. Microbiol. Sci. **4**:186–191.
30. **Platt, T.** 1986. Transcription termination and the regulation of gene expression. Annu. Rev. Biochem. **55**:339–372.
31. **Reimmann, C., R. Moore, S. Little, A. Savioz, N. S. Willetts, and D. Haas.** 1989. Genetic structure, function and regulation of the transposable element IS*21*. Mol. Gen. Genet. **215**:416–424.
32. **Roberts, R. C., R. Burioni, and D. R. Helinski.** 1990. Genetic characterization of the stabilizing functions of a region of broad-host-range plasmid RK2. J. Bacteriol. **172**:6204–6216.
33. **Rogowsky, P., and R. Schmitt.** 1985. Tn*1721*-encoded resolvase: structure of the *tnpR* gene and its in vitro functions. Mol. Gen. Genet. **200**:176–181.
34. **Sanger, F., S. Nicklen, and A. R. Coulsen.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
35. **Saurugger, P. N., O. Hrabak, H. Schwab, and R. M. Lafferty.** 1986. Mapping and cloning of the *par*-region of broad-host-range plasmid RP4. J. Biotechnol. **4**:333–343.
36. **Schwab, H., P. N. Saurugger, and R. M. Lafferty.** 1983. Occurrence of deletion plasmids at high rates after conjugative transfer of the plasmids RP4 and RK2 from *Escherichia coli* to *Alcaligenes eutrophus* H16. Arch. Microbiol. **136**:140–146.
37. **Seth, A.** 1984. A new method for linker ligation. Gene Anal. Tech. **1**:99–103.
38. **Sherratt, D.** 1989. Tn*3* and related transposable elements: site-specific recombination and transposition, p. 163–184. *In* D. E. Berg and M. M. Howe (ed.), Mobile DNA. American Society for Microbiology, Washington, D.C.
39. **Stirling, C. J., G. Steward, and D. J. Sherratt.** 1988. Multicopy plasmids stability in *Escherichia coli* requires host-encoded functions that lead to plasmid site-specific recombination. Mol. Gen. Genet. **214**:80–84.
40. **Summers, D. K., and D. J. Sherratt.** 1984. Multimerization of high copy number plasmids causes instability: ColE1 encodes a determinant essential for plasmid monomerization and stability. Cell **36**:1097–1103.
41. **Tabor, S., and C. C. Richardson.** 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc. Natl. Acad. Sci. USA **82**:1074–1078.
42. **Tabuchi, A., Y.-N. Min, C. K. Kim, Y.-L. Fan, D. D. Womble, and R. H. Rownd.** 1988. Genetic organization and nucleotide sequence of the stability locus of the IncFII plasmid NR1. J. Mol. Biol. **202**:511–525.
43. **Thomas, C. M., and D. Helinski.** 1989. Vegetative replication and stable inheritance of IncP plasmids, p. 1–25. *In* C. M. Thomas (ed.), Promiscuous plasmids of gram-negative bacteria. Academic Press, Inc. (London), Ltd., London.
44. **Tsuchimoto, S., H. Ohtsubo, and E. Ohtsubo.** 1988. Two genes, *pemK* and *pemI*, responsible for stable maintenance of resistance plasmid R100. J. Bacteriol. **170**:1461–1466.