JOURNAL OF BACTERIOLOGY, Dec. 1992, p. 8119–8132
0021-9193/92/248119-14$02.00/0

Vol. 174, No. 24

Definition of a Minimal Plasmid Stabilization System from the Broad-Host-Range Plasmid RK2

RICHARD C. ROBERTS† AND DONALD R. HELINSKI*

Department of Biology and Center for Molecular Genetics, University of California, San Diego, La Jolla, California 92093-0634

Received 19 June 1992/Accepted 15 October 1992

The stable inheritance of the broad-host-range plasmid RK2 is due at least in part to functions within a region located at coordinates 32.8 to 35.9 kb, termed the RK2 *par* locus. This locus encodes four previously identified genes in two operons (*parCBA* and *parD*; M. Gerlitz, O. Hrabak, and H. Schwab, J. Bacteriol. 172:6194–6203, 1990, and R. C. Roberts, R. Burioni, and D. R. Helinski, J. Bacteriol. 172:6204–6216, 1990). The *parCBA* operon is functional in resolving plasmid multimers to monomers. Analysis of the plasmid stabilization capacity of deletions within this region, however, indicates that this multimer resolution operon is required for stabilization only in certain *Escherichia coli* strains and under specific growth conditions. The deletion analysis further allowed a redefinition of the minimal functional region as 790 bp in length, consisting of the *parD* gene (243 bp) and its promoter as well as sequences downstream of *parD*. This minimal region stabilizes an RK2-derived minireplicon in several different gram-negative bacteria and, at least in *E. coli*, in a vector-independent manner. By insertional mutagenesis, both the *parD* gene and downstream (3′) regions were found to be required for plasmid stabilization. The downstream DNA sequence contained an open reading frame which was subsequently shown by transcriptional and translational fusions to encode a protein with a predicted size of 11,698 Da, designated ParE. Since the *parDE* operon requires the presence of the *parCBA* operon for efficient stabilization under certain growth conditions, the potential role of multimer resolution in plasmid stabilization was tested by substituting the ColE1 *cer* site for the *parCBA* operon. While the *cer* site did function to resolve plasmid multimers, it was not sufficient to restore stabilization activity to the *parDE* operon under growth conditions that require the *parCBA* operon for plasmid stability. This suggests that plasmid stabilization by the RK2 *par* locus relies on a complex mechanism, representing a multifaceted stabilization system of which multimer resolution is a conditionally dispensable component, and that the function(s) encoded by the *parDE* operon is essential.

RK2 is a large (60-kb), broad-host-range plasmid of the IncP1 family. It is stably maintained in *Escherichia coli*, in spite of its relatively low copy number of approximately 5 to 8 copies per chromosome (for a review, see reference 40). At such a copy number, it is likely that this plasmid encodes one or more elements responsible for ensuring its maintenance within the bacterial population (reviewed in references 2 and 29). Recent studies have shown that RK2 and the homologous isolate from *Pseudomonas* sp., RP4 (7), do contain information that promotes plasmid stabilization. A region within the *PstI* C fragment of these plasmids, from approximately 32.6 to 35.8 kb, has been shown to efficiently stabilize plasmids in a broad-host-range, vector-independent manner (18, 31, 33). This region, termed the RK2 or RP4 *par* region, encodes several genes that have been proposed to form two divergently transcribed operons, one containing (in order of transcription) *parC*, *parB*, and *parA*, and the other containing *parD* (see Fig. 1). Their promoters (P*parCBA* and P*parD*), located within a sequence of less than 200 bp, have been shown to be autoregulated by the ParA and ParD proteins, respectively (11, 15). The region encoding these promoters has also been shown to include the *cis*-acting site for *mrs* activity (31). The *parA* gene encodes a protein with considerable homology to the resolvases of the Tn*3* transposon family (18), and it has been shown that the *cis*-acting site along with proteins encoded by the *parCBA* operon is sufficient to catalyze resolution of plasmid multimers (18, 21, 31). This multimer resolution region, however, is not sufficient for plasmid stabilization; sequences in the *parD* operon region are required for efficient stabilization (18, 31). Other than the likely involvement of ParA in the resolution of multimers, the functions of the other gene products from these two operons are unknown. Since a *cis*-acting site capable of stabilizing a vector when the RK2 *par* proteins are provided in *trans* has not been identified to date (18, 31, 36), this region is generically designated as a stabilization region until further information is obtained to determine whether or not it specifies a true plasmid-partitioning activity.

In this report, we describe a plasmid-stabilizing activity within the 3.2-kb RK2 stabilization region that requires only 0.8 kb of DNA sequence information within the *parD* operon. This region is sufficient to confer vector-independent, broad-host-range stability under several conditions tested. However, in the case of rich-medium growth of *E. coli* carrying a wild-type *recA* gene, this minimal region is insufficient for plasmid stabilization and requires the presence of the *parCBA* operon. The DNA sequence of this 0.8-kb region was determined, and a new gene, *parE*, was identified immediately downstream of the *parD* gene. Expression of the 103-amino-acid ParE protein was demonstrated in vivo by using both transcriptional fusions under T7 promoter control and translational fusions to β-galactosidase. Mutational analysis of this region demonstrated that both *parD* and *parE* are required to effect plasmid stabilization. Under conditions in which the 0.8-kb region is insuffi-

* Corresponding author.
† Present address: Department of Developmental Biology, Stanford University School of Medicine, Stanford, CA 94305.

TABLE 1. Bacterial strains used

Strain	Relevant characteristics	Source
A. tumefaciens A348	Nal^r	17
A. vinelandii UW	Wild type	5
P. aeruginosa PAO1161	Spontaneous Nal^r mutant	M. Bagdasarian
E. coli		
TG1	(F' *lacI*^{q} *proA*$^+$*B*$^+$ *lacZ*ΔM15) *sup*^{0}	8
DH5	F$^-$ *recA1 gyrA96 supE44*	22
MC1061	F$^-$ *sup*^{0}	10
C600	F$^-$ *supE44*	1
GM1859	*himD*$^+$	12
GM1859*him3*	GM1859 *himD* (*cat* insertion in *himD*)	12
K37	*gal rps1 himA*$^+$	19
K2691	K37 Δ*himA* (*Sma*I deletion within gene)	19
RR1	F$^-$ *supE44 recA*$^+$	6
HB101	RR1 *recA13*	6
JM107	(F' *traD36 lacI*^{q} *proA*$^+$*B*$^+$ *lacZ*ΔM15) *supE44 gyrA96*	44
JM109	JM107 *recA1*	44
RK4536	*gyrA90*; CGSC 5850 (Yale University)	B. Bachmann
SS320	*gyrA19*; CGSC 6414 (Yale University)	B. Bachmann
DPB635	*topA*$^+$	4
DPB636	DPB635 *topA66*	4
LE234	*gyrB*$^+$	30
LE316	LE234 *gyrB*(Ts)	30
BL21	F$^-$ *hsdS gal*	37
DS941	*xerA*$^+$	38
DS941*xerA*	DS941 with a Tn*5* insertion in the *xerA* gene	D. K. Summers

cient for stabilization, multimer resolution by the ColE1 *cer* site was unable to substitute for the stabilizing activity of the *parCBA* operon. This indicates that plasmid stabilization by the RK2 *par* region is likely due to a multifunctional stabilization system of which the *parDE* operon is essential and multimer resolution is a conditionally dispensable element.

MATERIALS AND METHODS

Materials. Restriction endonucleases, Klenow fragment of *E. coli* DNA polymerase, T4 DNA polymerase, and T4 DNA ligase were obtained from several sources (Boehringer Mannheim Biochemicals, Indianapolis, Ind.; New England Biolabs, Beverly, Mass.; Pharmacia LKB Biotechnology, Pleasant Hill, Calif.; and Bethesda Research Laboratories, Gaithersburg, Md.) and were used as described in the manufacturer's specifications. The exonuclease III/mung bean nuclease deletion kit from Stratagene (La Jolla, Calif.) was used to generate nested deletions. [α-^{35}S]dATP (1,000 Ci/mmol) and [α-^{32}P]dATP (3,000 Ci/mmol) were purchased from New England Nuclear (Boston, Mass.). L-[^{35}S]methionine (1,000 Ci/mmol; in vivo labeling grade) was purchased from Amersham Corp. (Arlington Heights, Ill.). DNA sequencing was performed with Sequenase from U.S. Biochemicals (Cleveland, Ohio). Antibiotics were supplied by Sigma Chemical Co. (St. Louis, Mo.).

Strains and media. The bacteria used in this study and their sources are listed in Table 1. All *E. coli* strains were grown in either LB medium (GIBCO Scientific, Grand Island, N.Y.) or M9 medium (32) as indicated. *Pseudomonas aeruginosa* was also grown in LB medium. *Agrobacterium tumefaciens* was grown in YEP medium as described previously (31). *Azotobacter vinelandii* was grown in Burk medium (28). Antibiotics for *E. coli* selection were added at final concentrations of 250 μg/ml for penicillin, 40 μg/ml for kanamycin, and 10 μg/ml for tetracycline. Carbenicillin at 100 μg/ml was used for selection in *P. aeruginosa* and *A. tumefaciens*, whereas a concentration of 50 μg/ml was used for *A. vinelandii* selection.

Plasmid constructions. All DNA manipulations, including restriction enzyme digestions, filling in of 5′ overlapping ends by Klenow fragment, removal of 3′ overlapping ends by T4 DNA polymerase, DNA ligation, agarose gel electrophoresis, and *E. coli* transformation have been described (32). Plasmids used in this study are listed in Table 2.

Nested deletions of the sequence downstream of the defined *parD* gene were generated through exonuclease III digestion (32) as described in the protocol supplied by the manufacturer of the kit. The plasmid pRR41-8R (see Fig. 2) was first linearized at the downstream border by digestion with *Bam*HI and *Sph*I within the vector polylinker. This DNA was treated with exonuclease III to generate deletions by using the standard protocol (32). This strategy maintained the *Sac*I and *Kpn*I restriction sites of the vector adjacent to the previously defined 8R border (31) and placed the *Hin*dIII site adjacent to the newly deleted endpoint. Constructs carrying appropriate deletions were identified by DNA isolation and restriction analysis and then subcloned to pBluescript II SK(+) by using either the *Kpn*I and *Hin*dIII or *Sac*I and *Hin*dIII sites, to place the fragments in both orientations with respect to the vector. The endpoint of each new deletion was determined from the *Sac*I-to-*Hin*dIII derivatives by single-stranded DNA sequencing (32). The deleted regions were then available for subcloning as *Kpn*I-to-*Bam*HI fragments (from the *Kpn*I-to-*Hin*dIII derivatives), maintaining the same orientation of the *parD* gene with respect to the recipient vectors as that of previously tested deletions (31).

Of the deletion derivatives of plasmid pRR41-8R (diagrammed in Fig. 2), the Δ2 deletion best exhibited the properties of a minimal region fully functional in stabilization; it was designated pRR41-0.8. The pBluescript II SK(+) derivative carrying this deletion, subcloned as a *Kpn*I-to-*Hin*dIII fragment, was further modified by removal of the vector *Afl*III site by means of partial digestion with *Afl*III, Klenow filling in, and religation. This new construct was designated pRR120. Plasmid pRR120Δ6 was generated by replacing the Δ2 deletion fragment in pRR120 with the Δ6 deletion fragment (see Fig. 2).

Plasmid pRR120 next served as the substrate for linker insertion mutagenesis. Partial digestion with *Ava*II, *Bgl*I, *Dde*I, or *Esp*I was followed by elimination of 3′ (*Bgl*I sites) or 5′ (other sites) single-stranded ends and religation in the presence of either 8-bp phosphorylated *Bgl*I linkers (5′-pCAGATCTG-3′) or 12-bp nonphosphorylated *Nhe*I linkers (5′-CTAGCTAGCTAG-3′). The number of linkers inserted was determined by DNA sequence analysis: the *Nhe*I linkers were present only once at each site, while the *Bgl*II linkers were present in multiple copies. These extra *Bgl*II linkers were removed by extensive digestion with *Bgl*II, isolation of the DNA by agarose gel electrophoresis, and religation of the linear plasmid. Subsequent sequence analysis identified single linker insertions at all sites except the *Bgl*I site at the center of *parD*; the only viable constructs recovered still contained multiple linkers. For further analysis, the stabilization region of the pRR120 derivative carrying an *Nhe*I

TABLE 2. Plasmids used

Plasmid	Relevant characteristics	Source
pBluescript II SK(+)	Cloning vector	Stratagene
pRK2013	ColE1 replicon; RK2 Tra$^+$	13
pAL4000	RSF1010 vector; *tet*, *luc* reporter gene	20
pTD11	pAL4000 with P*parD* transcribing *luc*	11
pDK6	Protein overexpression vector	24
pGP1-2	p15A replicon carrying the T7 gene *1* RNA polymerase gene	39
pMC931	p15A replicon carrying *lacZ* translational fusion cassette	9
pKS490	pUC8 with the ColE1 *cer* site inserted at *Acc*I	38
pRR10	Mini RK2, *bla oriT lacZ*α, cloning and broad-host-range stabilization assays	31
pRR10-ts97	pRR10 with temperature-sensitive *trfA* replication gene	31
pRR15	Mini R6K, *npt lacZ*α	31
pRR54	pRR10 + RK2 *par* region (32.6 to 35.8 kb)	31
pRR61	pBK280 with F *ccd* region removed	31
pRR62	pRR61 + RK2 *par* region (32.6 to 35.8 kb)	31
pRR41-8R	pRR10-ts97 carrying the RK2 *Pst*I C fragment deletion 8R (34.7 to 37.0 kb)	31
pRR41-3.2	pRR10-ts97 + 3.2-kb RK2 *par* region (32.6 to 35.8 kb; *Sst*II to *Cla*I)	31
pRR41-0.8	pRR10-ts97 + 0.8-kb RK2 *par* region (34.7 to 35.5 kb)	This work
pRR41-0.8D	pRR41-0.8 with *Nhe*I linker inserted in *parD*	This work
pRR91	pRR10 + 0.8-kb RK2 *par* region, Δ2 border	This work
pRR91Δ6	pRR10 + 0.8-kb RK2 *par* region, Δ6 border	This work
pRR91D	pRR91 with *Nhe*I linker inserted in *parD*	This work
pRR93	pRR10-ts97 with a deleted 3.2-kb RK2 *par* region (32.6 to 35.5 kb; *Sst*II to Δ2)	This work
pRR93Δ6	pRR10-ts97 with a deleted 3.2-kb RK2 *par* region (32.6 to 35.5 kb; *Sst*II to Δ6)	This work
pRR93D	pRR93 with *Nhe*I linker inserted in *parD*	This work
pRR97	pRR61 + 0.8-kb RK2 *par* region, Δ2 border	This work
pRR97Δ6	pRR61 + 0.8-kb RK2 *par* region, Δ6 border	This work
pRR97D	pRR97 with *Nhe*I linker inserted in *parD*	This work
pRR120	pBluescript II SK(+) with 0.8-kb RK2 *par* region and vector *Afl*III site removed	This work
pRR120Δ6	pBluescript II SK(+) with 0.8-kb RK2 *par* region, Δ6 border	This work
pRR120D	pRR120 with *Nhe*I linker inserted in *parD*	This work
pRR121	pBluescript II SK(+) with *parD* and *parE* cloned downstream of the vector T7 promoter	This work
pRR136	pBluescript II SK(+) with *parD* cloned downstream of the vector T7 promoter	This work
pRR137	pBluescript II SK(+) with *parE* cloned downstream of the vector T7 promoter	This work
pRR138	pRR121 with *Bgl*II linkers inserted in *parD*	This work
pRR146-D	pRR120 with *lacZ* fused to *parD*	This work
pRR146-E	pRR120 with *lacZ* fused to *parE*	This work
pRR146-D#E	pRR120 carrying nine *Bgl*II linkers in *parD* and *lacZ* fused to *parE*	This work
pRR110	pBluescript II SK(+) with the ColE1 *cer* site inserted	This work
pRR116-1	pRR10-ts97 carrying the ColE1 *cer* site	This work
pRR116-2	pRR41-3.2 carrying the ColE1 *cer* site	This work
pRR116-3	pRR41-0.8 carrying the ColE1 *cer* site	This work

linker at the *parD Ava*II site (designated pRR120D) was transferred back to pRR10-ts97 as a *Kpn*I-to-*Bam*HI fragment, preserving its orientation relative to pRR41-0.8, to generate pRR41-0.8D.

Analysis of the properties of the defined 0.8-kb region required its transfer to two other vectors, namely, the mini-RK2 replicon pRR10 (for host range studies) and the mini-F replicon pRR61 (for vector independence studies). The 0.8-kb region from pRR120, pRR120Δ6, and pRR120D was in each case subcloned into both of these vectors by using the polylinker restriction sites *Kpn*I and *Bam*HI (maintaining their orientation in comparison with previously made derivatives). The resulting plasmids were designated pRR91, pRR91Δ6, and pRR91D, respectively, for the pRR10-based series, and pRR97, pRR97Δ6, and pRR97D, respectively, for the pRR61-based series.

Determination of whether the "right" border of the 0.8-kb region defined by exonuclease III deletion (see Fig. 2) also serves as the right border for the larger stabilization region encoding the *parCBA* operon required the transfer of these deletions to the plasmid pRR41-3.2. Such a transfer results in substitution of the new deletions for the previously defined *Cla*I border (31). The transfer was performed by using the unique internal restriction site *Afl*III (nucleotide [nt] 2299) within the stabilization regions of plasmids pRR41-3.2 and pRR120, pRR120Δ6, or pRR120D and the common external site *Bam*HI. Switching of the sequences downstream of *parD* was confirmed by analysis of restriction fragment sizes in this region. The clones carrying the intact 0.8-kb border, the Δ6 border, or the *Nhe*I linker-mutagenized region were labeled pRR93, pRR93Δ6, and pRR93D, respectively (see Fig. 2).

Detection of the protein products of the *parD* and *parE* genes was facilitated by placing these genes under the control of the T7 promoter present on pBluescript II SK(+) adjacent to the *Kpn*I site, with transcription proceeding into the polylinker. Expression of the entire region was accomplished by deletion of the native *parD* promoter via digestion of pRR120 with *Kpn*I and *Afl*III (nt 2299), making the ends flush with T4 DNA polymerase, and religation to form pRR121. Expression of the *parD* gene alone was achieved by removal of the *parE* sequences between the *Bgl*II linker inserted at the *Dde*I site at the 5′ end of *parE* (nt 2594) and the polylinker *Bam*HI site, followed by removal of the P*parD* sequence by cleavage with *Kpn*I and *Afl*III. This construct was designated pRR136. Expression of *parE* alone

was engineered by deletion of both P*parD* and the *parD* gene between the *Kpn*I site and the *Bgl*II linker at the *Dde*I site at the 3′ end of *parD* (nt 2555) to generate pRR137 (this construct leaves the native *parE* Shine-Dalgarno sequence intact). Finally, expression of an altered form of ParD and native ParE was achieved by deletion of a *Kpn*I-to-*Afl*III fragment containing P*parD* from a construct carrying nine *Bgl*II linkers at the *Bgl*I site at the center of *parD* (which should add 23 amino acids to ParD) to generate pRR138.

Synthesis of ParD and ParE was further studied by using translational fusions generated between *parD* or *parE* and the *lacZ* gene. The *lacZ* fusion cassette from plasmid pMC931 was recovered as a *Bam*HI fragment and inserted at the *Bgl*II linker either in the center of *parD* (at the *Bgl*I site, nt 2447) to generate pRR146-D or near the center of *parE* (at the *Esp*I site, nt 2674) to generate pRR146-E. Insertion of the *lacZ* cassette directly at the *Esp*I site within *parE* (carrying a *Bam*HI linker) in the pRR120 derivative carrying nine *Bgl*II linkers at the *parD Bgl*I site generated pRR146-D#E.

Plasmid pKS490 was used to determine whether the RK2 *mrs* region could be functionally replaced by the ColE1 *cer* site (and its required host cell components) (38). The *cer* site was first subcloned to pBluescript II SK(+) by using the restriction enzymes *Bam*HI and *Pst*I to generate pRR110. The *cer* site from this plasmid was then transferred from pRR110 to the plasmids pRR10-ts97, pRR41-3.2, and pRR41-0.8 by using the restriction sites *Sac*I and *Kpn*I, which lie between the *trfA* replication gene and the site of stabilization region insertion (at the same position that the RK2 *parCBA mrs* region occupies relative to the *parDE* operon in the intact region). The resulting plasmids were designated pRR116-1, pRR116-2, and pRR116-3, respectively.

Analysis of plasmid stability. The stabilization capacities of the 3.2-kb region and its deletion derivatives were determined as described previously (31). Briefly, for fragments cloned into the temperature-sensitive vector pRR10-ts97, a short-term overnight assay was used in which *E. coli* strains carrying the plasmids were grown to mid-log phase under antibiotic selection for the plasmids at 33.5°C, a temperature semipermissive for replication. At time zero, the cells were diluted into antibiotic-free medium and allowed to grow overnight at the same temperature to mid-log or stationary phase, depending on the *E. coli* strain used. Aliquots of cells were plated on antibiotic-free medium both before and after overnight growth, and the resulting colonies were tested for antibiotic resistance to determine the percentage of cells carrying plasmid at the initial and final time points. The percent loss per generation was calculated by using the formula:

$$1 - \left(\sqrt[n]{\frac{F_f}{F_i}} \right) \times 100 \qquad (1)$$

where n is the number of generations elapsed, F_i is the fraction of cells containing plasmid at the initial time point, and F_f is the fraction of cells containing plasmid at the final time point. The stabilization index for each construct was expressed as the ratio of the loss rate of pRR10-ts97 to the loss rate of this vector containing the fragment being analyzed, with indices averaged over multiple trials for each plasmid tested.

Stability analyses of clones based on the pRR10 or pRR61 vectors were performed in a modified manner. Since these vectors show only a low level of instability, the strains were maintained without antibiotic selection for 100 to 200 generations in log-phase growth, with plasmid loss assayed as described above.

Conjugal plasmid transfer. The plasmid pRR10 and its derivatives pRR54, pRR91, pRR91Δ6, and pRR91D were established in *A. tumefaciens*, *A. vinelandii*, and *P. aeruginosa* by triparental mating using pRK2013 as the source of the RK2 Tra factors as described previously (13). Exconjugants were selected by using nalidixic acid (20 μg/ml) and carbenicillin (100 μg/ml) for *A. tumefaciens* and *P. aeruginosa* or carbenicillin (50 μg/ml) for *A. vinelandii*.

Analysis of ParD-mediated repression of P*parD*. The capacity of the various linker insertion mutations within the 0.8-kb region to repress P*parD* was assayed as described previously (11). P*parD* is present on pTD11 such that it promotes expression of the luciferase gene. Either the intact 0.8-kb region or linker insertion mutations thereof were first subcloned as *Kpn*I-to-*Bam*HI fragments into the R6K-based vector pRR15. These plasmids were then established in *trans* to pTD11, and the effect on transcription of P*parD* was assessed by using a standard luciferase assay (20). Values obtained were compared with those of the unrepressed state (pRR15 in *trans*) or the fully repressed state (wild-type 0.8-kb region in *trans*).

Expression of Par proteins. The expression and radiolabeling of the *parD* and/or *parE* gene products first required establishment of the plasmids pBluescript SK(+), pRR121, pRR136, pRR137, and pRR138 in *E. coli* BL21 carrying pGP1-2 (to supply the T7 RNA polymerase). Crude cell extracts were prepared from these strains by growing them as described previously (39), using a 10-min 42°C heat shock on 10-ml cultures at an A_{600} of approximately 0.5 to induce the synthesis of the T7 RNA polymerase gene. Expression of cellular genes was inhibited by the addition of rifampin at a final concentration of 400 μg/ml; the cells were then grown for an additional 15 min at 42°C. Labeling with 10 μCi of [^{35}S]methionine per culture was performed during incubation at 30°C for 1 h to allow overproduction of the protein(s). The cells were harvested by centrifugation and then lysed in loading buffer and electrophoresed by using a 12% polyacrylamide gel based on the low-molecular-weight gel system of Schägger and von Jagow (34). Finally, the gel was fixed in 10% (vol/vol) acetic acid–45% (vol/vol) methanol, dried, and autoradiographed.

To detect the products of *parD*::*lacZ* or *parE*::*lacZ* fusions, *E. coli* TG1 strains carrying pRR120, pRR146-D, pRR146-E, or pRR146-D#E were grown to early log phase (A_{600}, 0.2 to 0.4). These cells were then assayed for the presence of β-galactosidase by using the procedure of Miller (27). Alternately, direct visualization of β-galactosidase fusions was demonstrated by first growing HB101 cells carrying pRR120, pRR146-D, pRR146-E, or pRR146-D#E to mid-log phase. The A_{600} of the culture was determined, and then the cells were harvested by centrifugation and lysed in loading buffer. On the basis of the A_{600} of the culture, predetermined amounts of cells were loaded and electrophoresed in a 6% polyacrylamide gel prepared by the method of Laemmli (25). The native β-galactosidase and the fusion proteins were then visualized by Western blot (immunoblot) of the proteins and reaction with anti-β-galactosidase antibodies by using the procedure described previously (14).

Analysis of plasmid multimer forms. The extent of multimerization of the vector pRR10-ts97 with or without the RK2 stabilization regions or the ColE1 *cer* site was determined by growing strains in either LB or M9 medium as indicated. The cells were grown at the semipermissive temperature of 33.5°C to mid-log phase with antibiotic selection, to mimic as

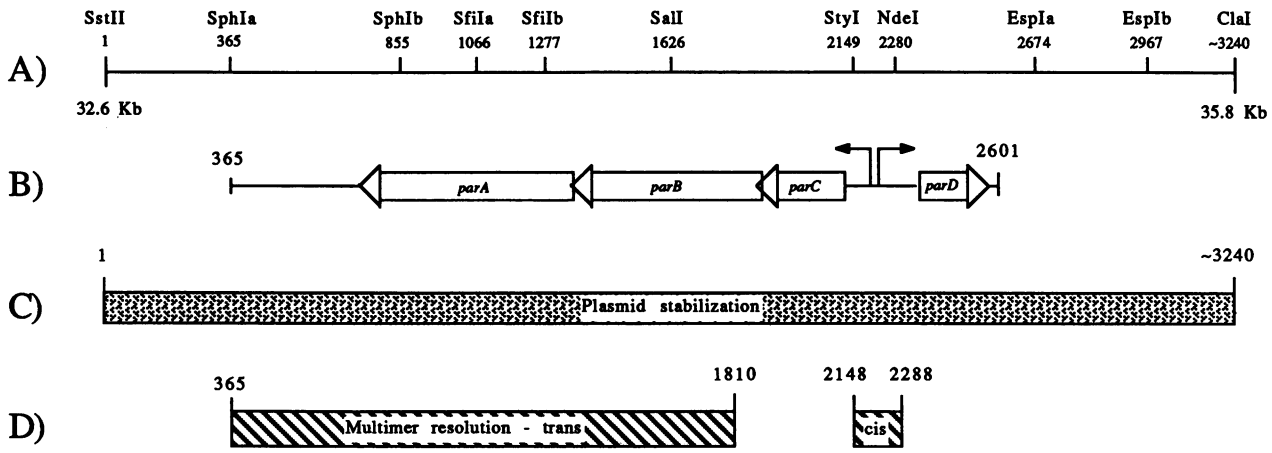

FIG. 1. The RK2 plasmid stabilization region. The defined region within the RK2 *Pst*I C fragment shown to promote efficient plasmid stabilization (18, 31) is diagrammed. Line A represents the pertinent restriction sites within the region. Numbers represent the first nucleotide of the restriction site, with nt 1 defined as the beginning of the *Sst*II site. Coordinates below the line indicate the position of the region within plasmid RK2, with the unique *Eco*RI restriction site defined as 0.0 kb (40). Line B represents the extent of the minimal stabilization region defined by Gerlitz et al. (18), with identified genes as well as the approximate location of the divergent promoters responsible for transcription of these genes shown (11, 15). Line C diagrams the minimal required region for efficient plasmid stabilization as previously defined in our laboratory (31). Line D indicates the locations of functions required for resolution of plasmid multimers, either supplied in *trans* (transcribed by an exogenous promoter) or in *cis* (31).

closely as possible the conditions employed during a short-term stabilization assay. The cells were harvested, their DNA was isolated, and approximately equivalent amounts of DNA were electrophoresed in a 0.8% agarose gel. The positions of different forms of the DNA were determined by Southern blotting as described previously (32). The hybridization probe used was the plasmid pAT50 (which carries the *Hae*II fragment of the RK2 origin of replication to specifically identify the RK2 minireplicons), which was linearized by digestion with *Eco*RI. The ends were then labeled with [α-^{32}P]dATP by Klenow fill-in as described previously (32).

Nucleotide sequence accession number. The nucleotide sequence reported for the 790-bp stabilizing sequence has been deposited with GenBank under accession number L05507.

RESULTS

Defining the minimal RK2 stabilization region. Previously, this laboratory determined by deletion analysis that the smallest portion of the RK2 *Pst*I C fragment that was capable of plasmid stabilization was 3.2 kb in size (31), spanning the region from the *Sst*II site to the *Cla*I site (Fig. 1). However, in subsequent experiments, these borders were found to be dependent on growth conditions. When deletions were tested in *E. coli* TG1 (the organism used in the original studies) grown in the defined M9 medium instead of the LB medium used previously, the region required for stabilization was substantially smaller. Deletions extending through the *parA*, *parB*, and *parC* genes which were previously unstable promoted efficient plasmid stabilization when the cells were grown in M9 medium. The new border on the *Sst*II side of the region was between the previously defined deletions 8R and 9R (31) (nt 2142 and approximately 2700, respectively [with the start of the *Sst*II site defined as nt 1]), so the 8R border was chosen as the "left" border of this smaller region (results not shown).

When the original deletions proceeding in from the right border (Fig. 1) were tested under these new growth conditions, the border previously observed remained unchanged, lying between the *Cla*I site (approximately nt 3240) and deletion 4L (nt 2542) (31). Partial digestion with *Esp*I and testing of these fragments for their ability to stabilize the vector pRR10-ts97 demonstrated that the 8R-to-*Esp*Ib fragment was functional while the 8R-to-*Esp*Ia fragment was not (Fig. 2). The precise border was more carefully defined by exonuclease III digestion of the region between *Esp*Ib and *Esp*Ia. The smallest region that promoted complete stabilization extended from deletion 8R (nt 2142; Fig. 2) to Δ2 (nt 2931), although deletion Δ5 (to nt 2853) retained some activity. The minimal stabilization region required in *E. coli* TG1 when grown in M9 medium is, therefore, 790 nt in length, much reduced from the ~3,200-nt fragment required when this strain is grown in LB medium. This new region was named the 0.8-kb stabilization region.

To determine whether the newly defined right border at nt 2931 was also functional in the presence of the *parCBA* operon, the *Cla*I border was substituted with either the Δ2 or the Δ6 deletions while retaining the region of the *parCBA* operon to the *Sst*II left border. When these constructs were tested in *E. coli* TG1 grown in LB medium, the Δ2 deletion efficiently stabilized while the Δ6 deletion did not (Fig. 2). This confirmed that the minimal right border lies between nt 2860 and 2931, regardless of the presence or absence of the *parCBA* operon.

Since it was possible that the stabilization capacity of the 0.8-kb region under these conditions is restricted to *E. coli* TG1, the pRR10-ts97 vector carrying either the 3.2- or 0.8-kb region was established in two other *recA*$^+$ *E. coli* strains, MC1061 and C600. Also tested were these regions with an *Nhe*I linker encoding translational termination codons in all three reading frames inserted at the *Ava*II site near the beginning of the *parD* gene (nt 2350), which should not be able to synthesize intact ParD protein. When these strains were tested for plasmid stability grown in either LB or M9 medium, results similar to those for *E. coli* TG1 were obtained (Table 3): the full-length region (nt 1 to 2931) stabilized completely in LB, while the deletion to Δ6 or

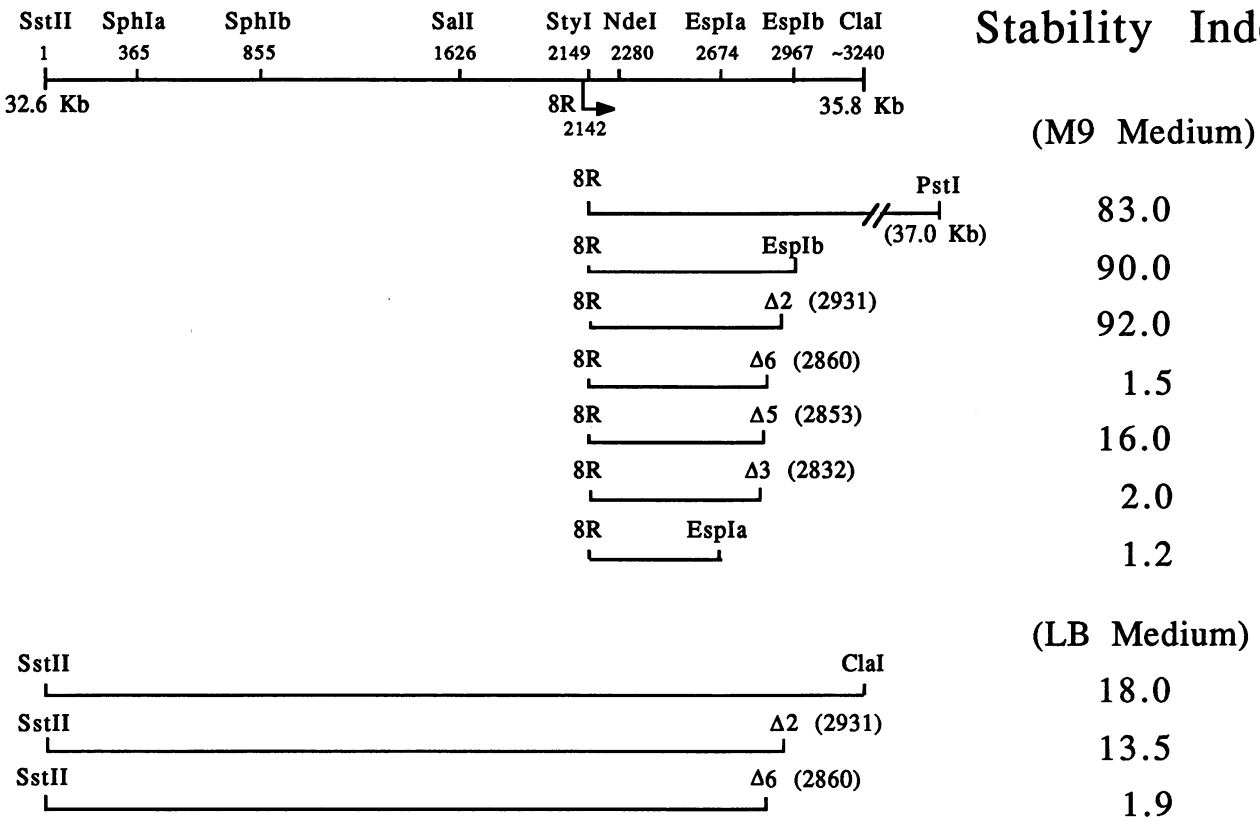

FIG. 2. Determination of sequences necessary for plasmid stabilization by deletion analysis. The 3.2-kb stabilization region and restriction sites are mapped on the top line. 8R designates a previous exonuclease III deletion endpoint which removed base pairs 1 to 2141 (31). Deletions from the *ClaI* end generated by restriction digestion or exonuclease III digestion along with their endpoints as identified by DNA sequencing are indicated. The assays were performed in *E. coli* TG1 in the indicated medium as described in Materials and Methods. The stabilization capacities of these deletions are reported as stability indices, calculated by dividing the percent loss rate of the vector (pRR10-ts97) per generation by that of the vector plus the diagrammed insert.

disruption of *parD* inactivated stabilization. The 0.8-kb region stabilized only very poorly in LB medium in each strain, while disruption of *parD* further lowered this residual activity. In contrast, growth in M9 medium allowed ParD-dependent stabilization by the 0.8-kb region in all strains.

While LB and M9 media differ greatly in chemical composition, three major components were tested independently in an attempt to identify the basis for the observed lack of plasmid stabilization by the 0.8-kb region in cells grown in LB medium. To more closely resemble the LB medium, the composition of the M9 medium was altered either by reduction of the concentration of $MgSO_4$ from 2 to 0.2 mM, supplementation with 0.2% (vol/vol) Casamino Acids, or supplementation with 0.45% NaCl. The 0.8-kb region stabilized efficiently under each of these modified conditions (results not shown). This suggests that neither the limiting Mg^{2+} concentration, the amino acid supplementation, the increasing growth rate, nor the higher ionic strength of the LB medium in comparison with that of M9 medium is responsible for the inability of the 0.8-kb stabilization region to function in LB medium.

TABLE 3. Stability of plasmids with alterations in the *parD* region in different *E. coli* strains

Medium and plasmid	Stability region (bases)	Stability index[a] in *E. coli* strain:		
		TB1	MC1061	C600
LB medium				
pRR93	1–2931	13.5	28.0	13.9
pRR93Δ6	1–2860	1.9	1.8	2.8
pRR93D	1–2931[b]	3.2	1.2	5.6
pRR41-0.8	2142–2931	3.8	6.9	4.3
pRR41-0.8D	2142–2931[b]	1.9	1.8	0.9
M9 medium				
pRR41-0.8	2142–2931	92.0	32.7	32.3
pRR41-0.8D	2142–2931[b]	1.8	1.2	0.9

[a] Stability index is defined as the loss rate of the vector divided by the loss rate of the vector carrying the indicated insert.
[b] Contains an *NheI* stop codon linker at the *AvaII* site of the *parD* gene.

Effect of host mutations on stabilization capacity of the 0.8-kb region. The capacity of the 0.8-kb region to stabilize a plasmid varied depending on the *E. coli* strain used; under the same growth conditions with LB medium, significant stabilization was seen in *E. coli* DH5 but not in *E. coli* TG1 (Table 4). Since these strains differ in multiple genetic loci and could not be directly compared, a study was initiated to examine 3.2- and 0.8-kb-region-mediated stabilization in several isogenic *E. coli* pairs. The loci chosen for study included the *himD* and *himA* genes encoding the subunits of IHF, which have been shown to play a role in the partitioning of the P1 prophage (16). The *recA* locus was chosen because of the potential destabilizing role of RecA-generated

TABLE 4. Stabilization of mini-RK2 by 3.2- and 0.8-kb *par* regions in different genetic backgrounds

Strain[a]	Relevant genotype[b]	Loss rate[c] (% per generation) of:		
		pRR10-ts97	pRR41-3.2	pRR41-0.8
TG1 grown in:				
LB medium	wt	9.60	0.30	2.53
M9 medium	wt	2.11	0	0
DH5	*gyrA96 recA1*	8.70	0.18	0.25
GM1859	wt	4.98	0.38	1.26
GM1859*him*	*himD*	10.60	0.04	0.15
K37	wt	1.95	0.29	0.97
K2691	*himA*	3.80	0.23	1.14
RR1	wt	5.14	0.44	4.11
HB101	*recA13*	1.59	0	0
JM107	wt	0.60	0	0.30
JM109	*recA1*	0.20	0	0
DPB635	wt	3.23	0.33	1.22
DPB636	*topA66*	0.31	0	0.04
RK4536	*gyrA90*	1.47	0	0
SS320	*gyrA19*	1.75	0.11	0.11
LE234 grown at:				
30°C	wt	0.30	0	0
33°C	wt	0.61	0	0
LE316 grown at:				
30°C	*gyrB*ts	0.63	0	0
33°C	*gyrB*ts	5.75	0	0.60

[a] All strains were grown in LB medium except where noted otherwise.
[b] wt, the isogenically wild-type strain of the pair.
[c] Efficient stabilization is defined when the loss rate with a stabilization locus present is 10% or less of the loss rate of pRR10-ts97.

multimers, which the 0.8-kb region would not be able to resolve (18, 21, 31). Finally, on the basis of the observed effects of superhelical density on stabilization of plasmid pSC101 (26), the role of in vivo superhelicity was investigated by using *gyrA*, *gyrB*, and *topA* loci.

The vector used in all cases was pRR10-ts97, and all strains were grown in LB medium. The results, summarized in Table 4, were obtained with isogenic pairs with the exception of strains TG1 and DH5 and the *gyrA* mutations. It should be noted that the loss rate of the vector pRR10-ts97 differed significantly among strains that were grown under the same conditions. The cause of this differing instability could not be clearly linked to any genotypic differences among the strains. The 3.2-kb stabilization region was found to promote efficient stabilization in all strains tested. The 0.8-kb region, however, was not able to efficiently stabilize the IHF isogenic wild-type strains but was functional in the presence of a *himD* mutation and only marginally active in a *himA* mutant background. In the case of the *recA* isogenic pairs, a mutation of *recA* allowed efficient stabilization for both alleles tested. The 0.8-kb region stabilized the pRR10-ts97 plasmid in all three strains carrying a DNA gyrase mutation and was proficient for stabilization in strain DPB635 only when the *topA* mutation was present.

Vector independence of the 0.8-kb stabilization region. The replicon independence of the 0.8-kb stabilization region was tested by inserting this sequence into the mini-F vector pRR61 along with corresponding defective regions carrying either the Δ6 deletion or the *Nhe*I linker in *parD*. When these derivatives were compared in the *recA E. coli* strain DH5 grown in LB medium, the intact 0.8-kb region showed only slight instability over 100 generations of continuous log-phase growth (plasmid present in 96% of the cells versus 100% retention for the 3.2-kb region), while the Δ6 deletion or disruption of *parD* increased the loss rate to the level seen for pRR61 alone (plasmid present in only 20 to 30% of the cells after 100 generations; results not shown). This demonstrated that the 0.8-kb region was not dependent on the RK2 replicon for its plasmid stabilization capacity in *E. coli*.

Broad-host-range activity of the 0.8-kb stabilization region. The possibility that the information encoded by the 3.2-kb region but lacking in the case of the 0.8-kb region is essential for broad-host-range function was tested by inserting either the wild-type 0.8-kb region or the Δ6 or *Nhe*I *parD* derivatives into pRR10. The stabilities of these plasmids in *E. coli* TG1, *P. aeruginosa* PAO1161Nal, *A. vinelandii* UW, and *A. tumefaciens* A348 in the absence of antibiotic selection were compared with those of pRR10 and pRR54 (pRR10 carrying the 3.2-kb region) (Fig. 3). *E. coli* TG1 and *P. aeruginosa* grown in LB medium demonstrated very similar loss kinetics. A slight difference was evident between the 3.2- and 0.8-kb regions in both organisms, but each region functioned better than the defective 0.8-kb regions or the vector alone. Both *A. vinelandii* and *A. tumefaciens* demonstrated no difference in plasmid stabilization between the 3.2- and 0.8-kb regions, while the defective regions generally resembled pRR10 alone in their loss kinetics. Thus, the 0.8-kb region is sufficient to promote a high degree of plasmid stabilization in a variety of gram-negative bacteria.

Regions of the 0.8-kb region important for stabilization as defined by insertional mutagenesis. To further define which sequences within the 0.8-kb region are necessary for stabilization, mutagenesis was performed by the insertion of linkers at multiple sites within the region. Seven sites of insertion were chosen (Fig. 4). Eight-nucleotide *Bgl*II linkers were inserted at all seven locations (either with single or multiple copies of the linker inserted, except as noted). In addition, 12-nt *Nhe*I linkers encoding translational stop codons in all three reading frames were inserted at the three sites within *parD*. The net number of bases inserted for each of the mutants as confirmed by DNA sequence analysis is indicated in Fig. 4.

The effect of these alterations on the stabilization activity of the 0.8-kb region was determined by testing their ability to stabilize pRR10-ts97 in *E. coli* DH5 grown in LB medium. Strain DH5 was chosen because of the high instability of the vector pRR10-ts97 in this organism (Table 4). The results (Fig. 4) demonstrated that the *Bgl*I site in the divergent promoter region is insensitive to the insertion of a 5-bp sequence. Within *parD*, any alteration of the gene at the 5′ end disrupted stabilization. Surprisingly, insertion or deletion of codons at the *Bgl*I site near the center of the gene had no effect on stabilization ability. At the 3′ end of the *parD* gene, alteration of the reading frame diminished stabilization activity while the insertion of stop codons eliminated such activity. It was noted, however, that *E. coli* DH5 carries the *supE44* mutation, which will allow partial read-through of the TAG stop codons inserted within *parD* by the *Nhe*I linkers. Therefore, the constructs carrying this linker at 5′ or 3′ locations were also tested in *E. coli* MC1061 in M9 medium, which is sup^0 (pRR41-0.8 carrying an *Nhe*I linker at the *Bgl*I site within *parD* could not be established in MC1061). The results remained unchanged (not shown). Finally, insertions at any of three locations within the sequence downstream of *parD* destroyed stabilization activity.

It was demonstrated that the region from deletion 8R to *Esp*Ia (Fig. 2) encodes the capacity to repress its own promoter when present in *trans* (11, 15). Further, insertion of an *Nhe*I linker at the 5′ end of *parD* disrupts autorepres-

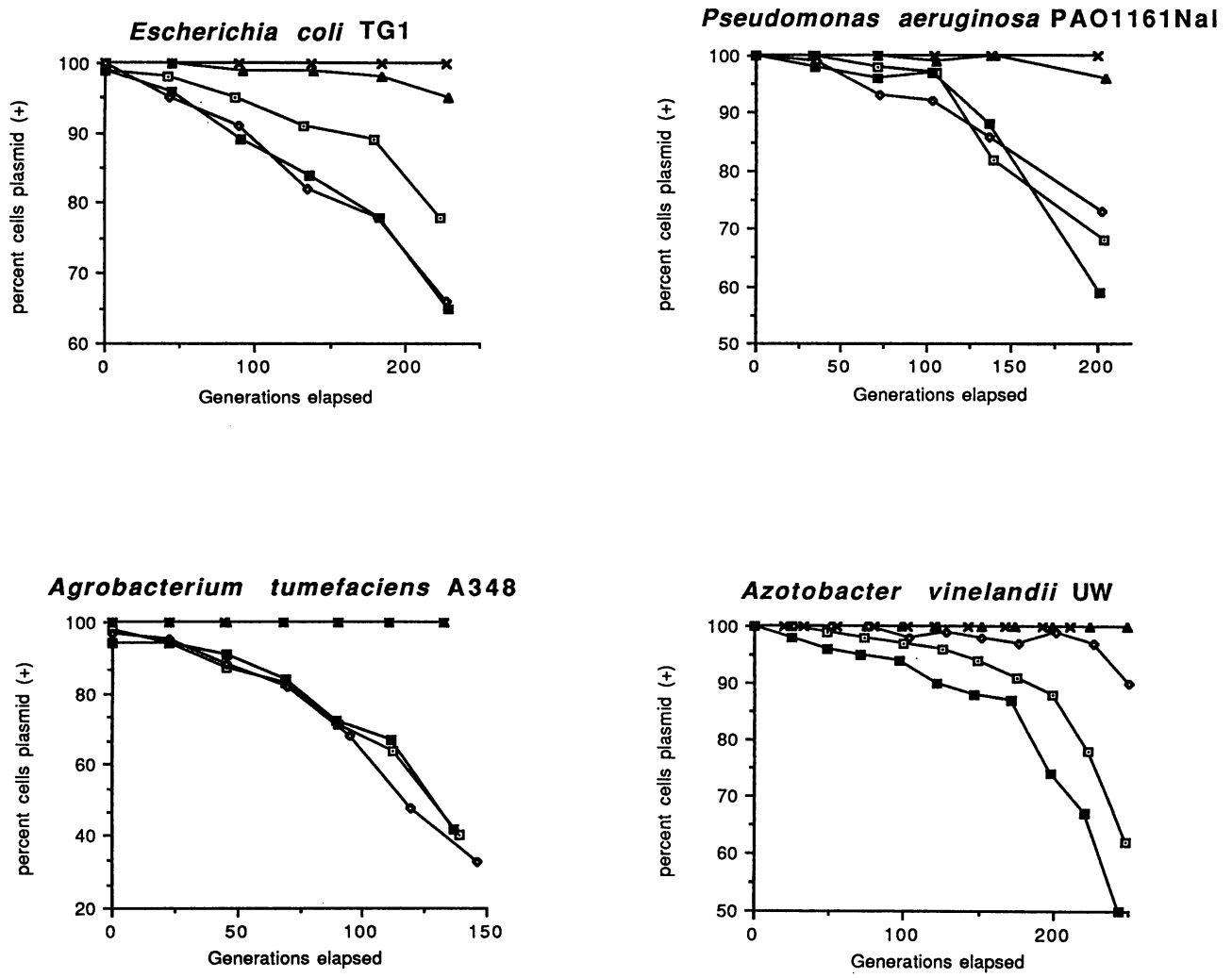

FIG. 3. Broad-host-range stabilization properties of derivatives of the 0.8-kb region. The ability of the 0.8-kb region or mutated derivatives to stabilize plasmids in diverse gram-negative organisms in comparison with that of the originally defined 3.2-kb region was analyzed in the mini-RK2 vector pRR10. These plasmids were established in *E. coli*, *P. aeruginosa*, *A. vinelandii*, and *A. tumefaciens* and assayed in rich growth medium as described in Materials and Methods for plasmid loss during continuous log-phase growth under nonselective conditions. Symbols: ⊡, vector pRR10; X, the 3.2-kb region; ▲, the wild-type 0.8-kb region; ◇, the defective 0.8-kb region deleted to Δ6; ■, the 0.8-kb region with translational stop codons inserted at the 5′ end of *parD*.

sion, indicating that the factor responsible is the ParD protein (11). To potentially define domains within the ParD protein, the insertional mutants of the 0.8-kb region were also tested for their ability to repress in *trans* the *parD* promoter transcribing the luciferase gene on pTD11. These results (Fig. 4) further indicated that ParD is the mediator of autorepression, since insertion of base pairs at the 5′ end or in the center of *parD* inactivated promoter repression, while insertion of bases upstream or downstream of the gene had no effect on repression. The addition of bases at the 3′ end of the gene had only minor effects. Finally, disruption of the *parD* promoter by filling in the *Nde*I site between the −10 and −35 boxes (15) or by the addition of a 12-bp *Nhe*I linker at this site also eliminated the ability to repress P*parD* in *trans* (results not shown). These data indicate that ParD is the only mediator of repression and that C-terminal amino acids of ParD, or elements downstream of this gene that are important for stabilization, are not required for autorepression.

When *Bgl*II linkers were inserted at the center of the *parD* gene, the only isolates obtained carried nine or three linkers. Isolates that carried a number of nucleotides that would result in a frameshift mutation were not recovered. Insertion of stop codons in all three reading frames by using the *Nhe*I linker was successful, but the plasmid could be established only in strains carrying a *supE* mutation (which would partially suppress the stop codons). It appears, therefore, that expression of ParD with an altered or truncated C-terminal region is toxic to the cells. Synthesis of wild-type ParD within the same cell as the truncated derivative has been observed to eliminate this toxicity (results not shown).

Characterization of the sequence downstream of the *parD* gene. It was found that deletions of the sequence downstream of the *parD* gene disrupted stabilization capacity when the sequence between nt 2931 and nt 2860, or beyond, was removed (Fig. 2) and that insertion of linkers at any of three locations within this downstream region also inactivated the stabilization mediated by the 0.8-kb region (Fig. 4).

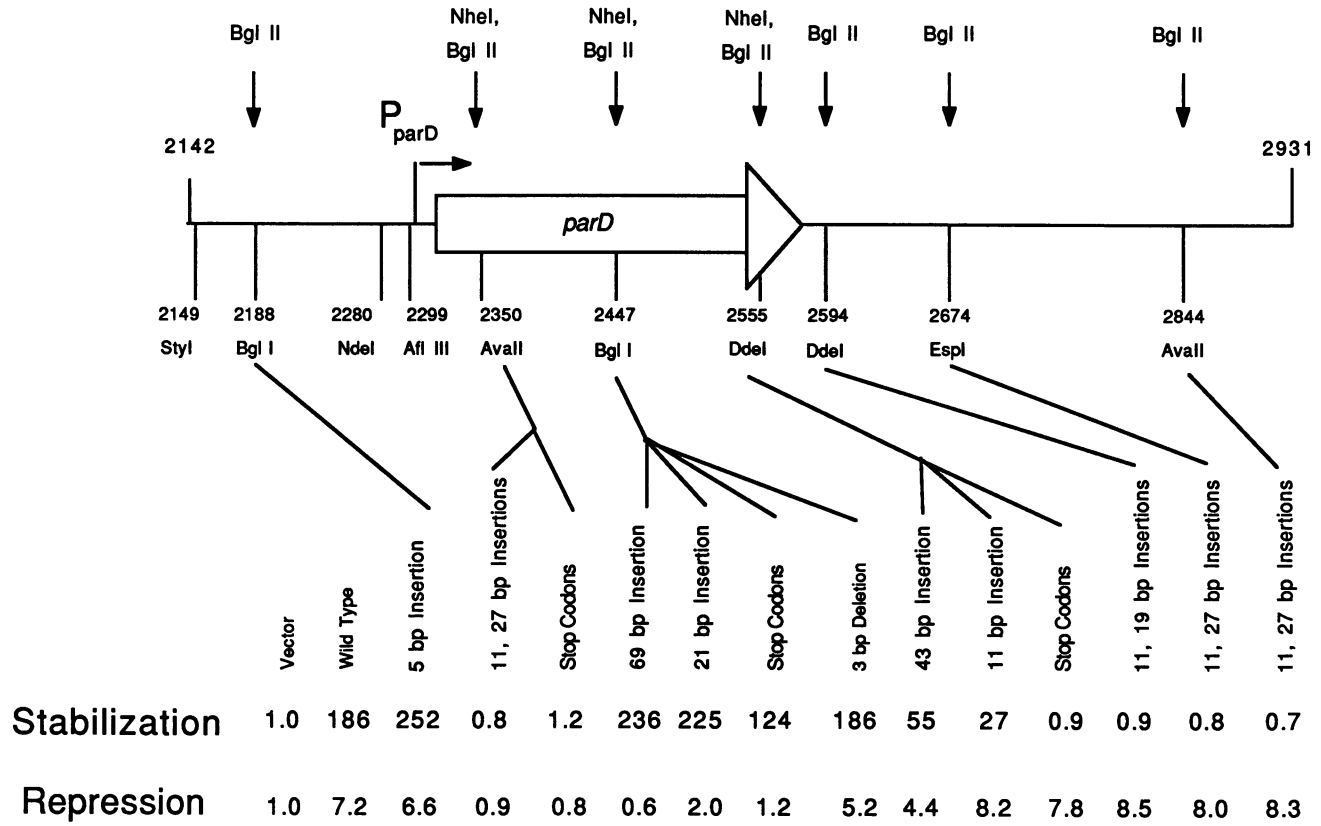

FIG. 4. Insertional mutagenesis of the 0.8-kb stabilization region. The restriction map of the 0.8-kb region along with the locations of the *parD* gene and the P*parD* promoter is shown. Nucleotide numbers indicate the beginning of the listed restriction sites, based on numbering starting at the *Sst*II site, as shown in Fig. 1. Arrows above the map indicate the positions of oligonucleotide linker insertions, with *Bgl*II and *Nhe*I representing 8- and 12-nt linkers, respectively, inserted at these sites. The net number of nucleotides inserted or deleted as determined by DNA sequence analysis is indicated below the restriction map. The stabilization capacities of these mutants were tested in the vector pRR10-ts97 and are reported as stabilization indices (the percent loss rate of pRR10-ts97 per generation divided by that of this plasmid plus the insert). The ability of these mutants to repress the P*parD* promoter in *trans* on the plasmid pTD11 (transcriptionally fused to the *luc* reporter gene [11]) is reported as the ratio of luciferase activity generated by the *luc* vector pAL4000 to the luciferase activity of pTD11 with wild-type or mutated 0.8-kb regions present in *trans*.

Therefore, the sequence of the region downstream of the *Esp*Ia site was determined. When this sequence was combined with previously published information (18) and compared with an unpublished sequence from the laboratory of H. Schwab (35), a novel open reading frame was located between nt 2572 and nt 2883, beginning with a TTG start codon that overlaps the stop codon of *parD* (Fig. 5). The predicted protein is 103 amino acids in length and was tentatively designated ParE.

To determine whether this open reading frame was translated in vivo, expression of both *parD* and *parE* was tested under the control of the T7 promoter present in pBluescript II SK(+) by using the conditions previously described (39). When both *parD* and *parE* genes were present on the plasmid, protein bands with apparent M_rs of 9,000 and 11,000 appeared (Fig. 6A), corresponding to the predicted sizes of ParD and ParE (9,103 and 11,698, respectively). When the *parD* gene alone was present, only the smaller protein was expressed. Expression from a construct carrying an extra 23 codons within *parD* caused a specific shift of this lower band to a position above the putative ParE band, confirming that the protein with the apparent M_r of 9,000 is indeed ParD. When the proposed *parE* gene alone was placed under T7 promoter control, no specific labeled protein was observed (Fig. 6A, lane 5). In view of the overlapping arrangement of the *parD* and *parE* genes, this may indicate that *parE* expression requires translational coupling to *parD*.

An alternate approach to determining whether an open reading frame is expressed is through the use of translational fusions to β-galactosidase. The *lacZ* gene was fused to either *parD* (at the *Bgl*I site) or *parE* (at the *Esp*I site) under transcriptional control of the native *parD* promoter. When these constructs were established in *E. coli* TG1, the *parD*::*lacZ* fusion generated an average of 4,808 Miller units; the *parE*::*lacZ* fusion averaged 159 Miller units of activity. It is likely that the expression of the *parE*::*lacZ* fusion is low because of the translation of an active ParD protein, which would repress P*parD* (11, 15). To circumvent this, *lacZ* was fused at the *parE Esp*I site in the construct that carries 23 extra codons within *parD*, which inactivates ParD-mediated autorepression (Fig. 4). When the β-galactosidase activity of this construct was measured in *E. coli* TG1, an average of 555 Miller units was detected, indicating that inactivation of autorepression by ParD does enhance expression of ParE.

Final evidence for the translation of *parD* and *parE* was the demonstration that the *lacZ* fusion proteins were increased in size compared with native β-galactosidase.

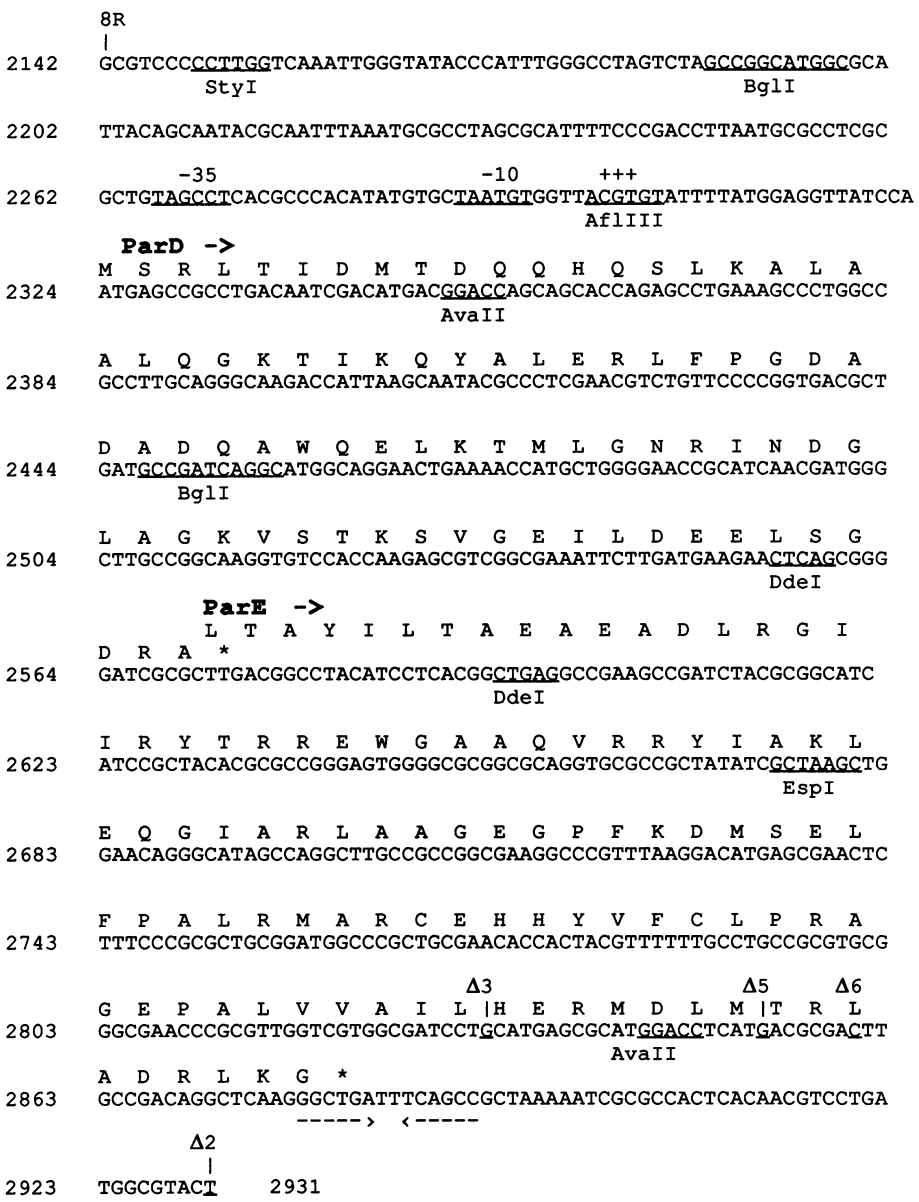

FIG. 5. Sequence of the 0.8-kb stabilization region. The nucleotide sequence of the minimal stabilization region is shown. Numbering begins at 2142, the first base of the 8R deletion (based on numbering from the *Sst*II site, as shown in Fig. 1). Pertinent restriction sites, as well as the previously identified transcriptional start sites for the *parD* promoter (represented by the plus symbols) (11, 15) and the endpoints of the 3′-end deletions generated in this study, are shown. The predicted amino acid sequences of the ParD and ParE proteins are also shown.

Expression of these fusions in *E. coli* HB101, followed by electrophoresis of the proteins and visualization by using anti-β-galactosidase antisera, showed the existence of proteins that were approximately 4,000 Da larger than the native β-galactosidase from this strain for both *parD*::*lacZ* and *parE*::*lacZ*, as predicted from the positions of the fusions (Fig. 6B). This evidence, coupled with the β-galactosidase activity and in vivo protein labeling, demonstrates that both the *parD* and *parE* genes are expressed and specify protein products.

Role of multimer formation and resolution in plasmid stabilization. The above observations show that the *parD* and *parE* genes constitute a plasmid stabilization system that functions independently from the *parCBA* operon in many but not all situations. The *parCBA* operon is known to encode an efficient multimer resolution system (18, 21, 31). Therefore, the simplest explanation for the failure of the 0.8-kb region to stabilize plasmids in all contexts is that under such conditions, multimeric forms predominate and interfere with stabilization. The *parCBA* operon would correct this by resolving such multimers to monomers. This hypothesis is consistent with the observation that the *parDE* operon functions in *recA* mutant hosts grown in LB medium, whereas stabilization is quite limited in isogenic *recA*$^+$ hosts in which the RecA protein acts to generate plasmid multimers (23). To test this hypothesis, initially the ColE1 *cer* site and the corresponding resolvase encoded by the bacterium were employed to functionally substitute for the RK2 *mrs*

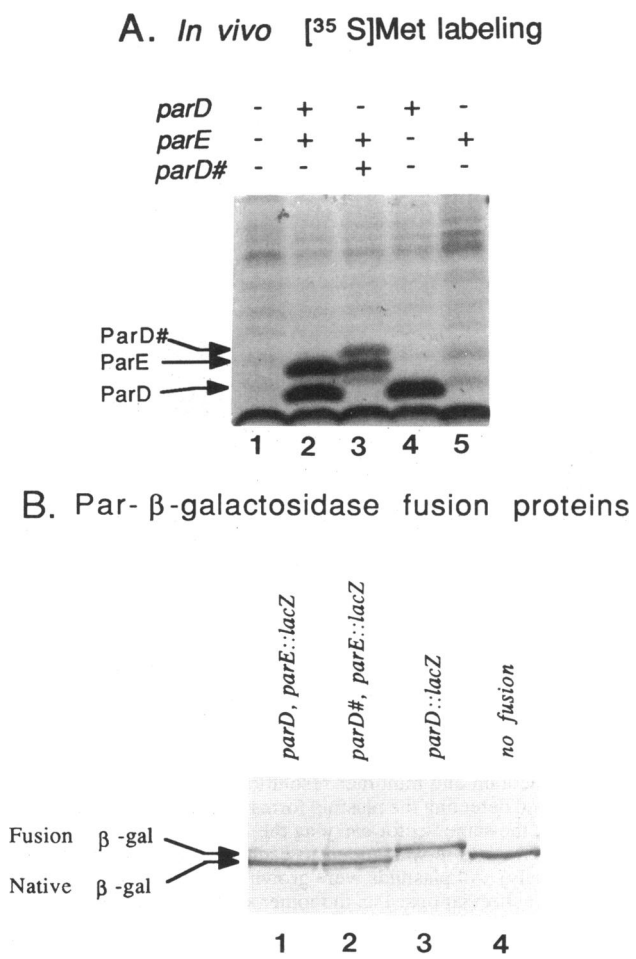

FIG. 6. In vivo expression of *parD* and *parE* genes. (A) Radioactive labeling of expressed proteins. *E. coli* BL21 strains carrying pGP1-2, to supply the T7 polymerase, and plasmids that encode *parD* and *parE* in different combinations under T7 promoter control were labeled in vivo with L-[^{35}S]methionine as described previously (39). The equivalent of 0.25 A_{600} unit of cells from these reactions was lysed and electrophoresed in a low-molecular-weight polyacrylamide gel (34) and then autoradiographed. Lanes: 1, pBluescript II SK(+) (no *par* genes); 2, pRR121 (*parD* + *parE*); 3, pRR138 (altered *parD*, *parE*); 4, pRR136 (*parD* only); 5, pRR137 (*parE* only). (B) Identification of β-galactosidase fusions with *par* proteins. *E. coli* HB101 cells carrying fusions between *parD* or *parE* and *lacZ* were harvested at early log phase, and the cell lysate in the amounts listed below was electrophoresed on a 6% polyacrylamide gel. Lanes: 1, 0.25 A_{600} unit of pRR146-E-containing cells (*parE*::*lacZ*); 2, 0.25 A_{600} unit of pRR146-D#E-containing cells (*parE*::*lacZ* with repressor defective *parD* to boost the level of *parE* expression); 3, 0.06 A_{600} unit of pRR146-D-containing cells (*parD*::*lacZ*); and 4, 0.15 A_{600} unit of pRR120-containing cells (no fusion). The β-galactosidase fusions were identified by Western blotting by using anti-β-galactosidase antibodies and alkaline phosphatase visualization. Each extract contained wild-type β-galactosidase in addition to the ParD- or ParE–β-galactosidase fusions. The calculated sizes of the ParD– and ParE–β-galactosidase fusions are in each case 120 kDa.

region. The *cer* site was, therefore, introduced into the vector pRR10-ts97 with or without the 3.2- or 0.8-kb stabilization region. Once established in *E. coli* TG1, these strains were grown in LB or M9 medium at 33.5°C (to mimic stabilization assay conditions). The plasmids were isolated and examined by Southern blotting to determine their multimer state (Fig. 7). A moderate percentage of multimers existed for plasmids that lacked a multimer resolution system (pRR10-ts97 or pRR41-0.8; Fig. 7, lanes 1 and 7) and the addition of either the ColE1 *cer* site (lane 3) or the RK2 *mrs* region (lane 4) resolved all of these multimers to monomers. If multimerization of pRR41-0.8 is responsible for its preferential instability in LB medium, then multimers of this plasmid should be present in TG1 cells grown in LB medium but absent in cells grown in M9 medium. A comparison of pRR10-ts97-, pRR41-3.2-, and pRR41-0.8-containing cells grown in LB medium with those grown in M9 medium (Fig. 7, lanes 1 and 2, 4 and 5, and 7 and 8, respectively) showed no M9 medium-dependent decrease in the level of multimerization.

Since the ColE1 *cer* site was observed to efficiently resolve pRR41-0.8 multimers, its ability to functionally substitute for the native RK2 *mrs* region was tested. When tested for its ability to stabilize pRR10-ts97 in either *E. coli* TG1 or DH5 (both grown in LB medium), the *cer* site had no effect (Table 5). The intact 3.2-kb region stabilized pRR10-ts97 completely, either with or without the *cer* site. Among these strains, pRR41-0.8, or the corresponding plasmids carrying the *cer* site, did not differ in stability. These plasmids were further tested in *E. coli* DS941 and DS941*xerA*; the latter strain carries a defective chromosomal *xerA* gene that renders the *cer* multimer resolution system inactive (38). No difference in stabilization of plasmids carrying the *cer* site was observed between these two strains whether or not the RK2 3.2- or 0.8-kb stabilization region was present (results not shown). This further demonstrates that *cer*-mediated multimer resolution does not enhance the stabilization capacity of the 0.8-kb region. Thus, the *parCBA* operon appears to contribute more to plasmid stabilization than simply resolution of multimers to monomers.

DISCUSSION

In this article, we describe the delineation of a segment within the 3.2-kb stabilization region of the plasmid RK2 that can efficiently stabilize plasmids in *E. coli* cells growing in minimal medium. This region, 0.8 kb in size, does not increase the copy number of the vector, is not capable of resolving plasmid multimers to monomers, and does not promote the killing of *E. coli* TG1 cells that have lost the plasmid when present in the context of the 3.2-kb region (31). The 0.8-kb region is able to function in a vector-independent manner and in a variety of gram-negative organisms, suggesting that it should be of general use in stabilizing plasmids for many applications. Recent experiments have shown that either the 3.2- or 0.8-kb region is sufficient to stabilize a mini-RK2 replicon in *Rhizobium meliloti* when the bacterium is growing either vegetatively or in planta (bacteria isolated from alfalfa nodules stably maintain a mini-RK2 replicon when the 0.8-kb region is inserted but not in its absence [43]).

The 3.2-kb region stabilizes plasmids in all *E. coli* strains tested, regardless of their genotype, but the genetic background of the *E. coli* strain when grown in LB medium plays a significant role in determining whether the 0.8-kb region can function or not. One major determinant appears to be the *recA* gene; if the wild-type gene is absent, then the 0.8-kb region is sufficient for plasmid stabilization (Table 4). The *recA* gene product blocks 0.8-kb region-mediated stabiliza-

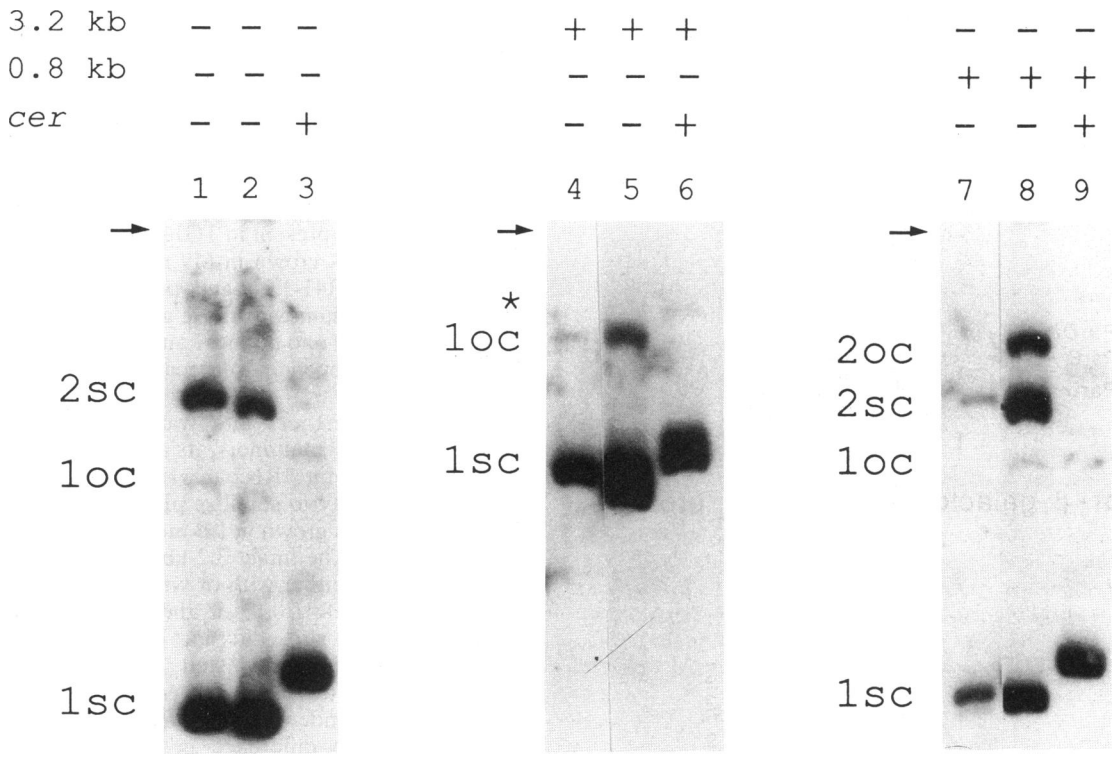

FIG. 7. Degree of pRR10-ts97 plasmid multimerization. The effects of growth medium and multimer resolution regions on the multimer state of the vector pRR10-ts97 were examined by isolating plasmid DNA from cells and detecting the plasmid forms by using Southern blotting as described in Materials and Methods. Lanes: 1 and 2, vector pRR10-ts97; 4 and 5, the same vector carrying the 3.2-kb stabilization region; 7 and 8, this vector carrying the 0.8-kb stabilization region. Additionally, the ColE1 *cer* site was added to each of the above plasmids to generate pRR116-1, pRR116-2, and pRR116-3 (shown in lanes 3, 6, and 9, respectively). All plasmids were grown in *E. coli* TG1 with either LB medium (lanes 1, 3, 4, 6, 7, and 9) or M9 medium (lanes 2, 5, and 8). Plasmid form abbreviations: 1sc, monomer supercoiled; 1oc, monomer open circular; 2sc, dimer supercoiled; 2oc, dimer open circular. Significant amounts of forms greater than dimer in size were not observed. Arrows indicate the position of sample loading, and the asterisk indicates the expected position of supercoiled dimers of pRR10-ts97 carrying the 3.2-kb region.

tion in a manner unrelated to multimer formation, since plasmid multimers are not preferentially formed in rich medium in *recA*$^+$ strains (Fig. 7).

The stabilization of several plasmids by an in vivo increase in negative superhelicity has been reported previously (26). The mini-RK2 replicon pRR10-ts97 behaves similarly, decreasing 10-fold in loss rate in the presence of the *topA66* mutation. However, complete stabilization of this vector in this strain required the presence of either the 3.2- or 0.8-kb RK2 stabilization region (Table 4). If superhelicity were the main effector of plasmid stabilization (as it appears to be for the plasmid pSC101 [26]), then a reduction in negative superhelicity as occurs in strains with DNA gyrase mutations should reduce stabilization ability. In *gyrA* or *gyrB* strains, no significant loss of plasmid stabilization was observed for either the 3.2- or the 0.8-kb region, suggesting that increased negative superhelicity is not the main component by which these regions achieve plasmid stability.

TABLE 5. Effect of ColE1 *cer* locus on activity of the 3.2- or 0.8-kb RK2 stabilization regions

Plasmid	Stability index[a] in:			
	E. coli TG1		*E. coli* DH5[b]	
	−*cer*	+*cer*	−*cer*	+*cer*
Vector alone	1.0	1.0	1.0	1.0
Vector + 3.2-kb stabilization region	26.0	21.5	174.0	156.0
Vector + 0.8-kb stabilization region	4.4	4.2	20.1	21.8

[a] Stability index is defined as the loss rate of the vector divided by the loss rate of the vector with the indicated region inserted. Strains were all grown in LB medium.

[b] Increased temperature conditions were employed, which decreased stabilization in DH5 by the 0.8-kb locus.

In the characterization of the 0.8-kb region, a new gene, *parE*, was identified as important for stabilization. This gene was shown to encode a protein with a predicted molecular weight of 11,698. Computer analysis of the ParE amino acid sequence indicates that the protein should have a pI of 9.4, mainly because of the presence of 13 arginine residues of the 103 total amino acids. A search of the EMBL and NBRF-Pir data bases revealed no proteins with significant amino acid homology. It is of interest that when Gerlitz et al. originally analyzed the corresponding RP4 *par* region for expressed gene products (18), a protein with an M_r of approximately 11,000 was found for clones carrying the *parE* gene. This gene product, tentatively assigned to the *parC* open reading frame (Fig. 1), conceivably could have also been the ParE protein. The latter possibility would explain the absence of this protein from expression products of the clone pGMA28, which carries an intact *parC* gene but lacks the *parE* gene (18).

The gene encoding the ParE protein plays no role in repression of the P*parD* promoter, as previously observed (11, 15), but was found to be necessary for efficient plasmid stabilization, in the context of either the 3.2- or the 0.8-kb region (Table 3). Insertion of extra base pairs at three internal sites within the *parE* gene, or deletion of sequence downstream of the Δ6 position (which changes the last five codons of the gene; Fig. 5), blocked stabilization activity under all conditions tested (Table 3 and Fig. 3). The further removal of 7 bp upstream of the Δ6 endpoint (to Δ5) partially restored stabilization capacity (Fig. 2), perhaps because of the proper positioning of fortuitous vector sequences adjacent to the Δ5 deletion. It is unknown how much of the 48-bp sequence downstream of the *parE* coding region between the end of the *parE* gene and the Δ2 deletion is necessary; this sequence may be responsible for ensuring the stability and proper termination of the mRNA, as reported for other transcripts (3).

The essentiality of the *parE* sequence is not consistent with the results of Gerlitz et al. (18), who found that the *parE* sequence could be deleted without loss of stabilization. These results were based on stabilization of a pBR322-based plasmid which was not tested in our studies. Stabilization by a truncated RK2 region lacking *parE* may be due to the higher intrinsic copy number of this vector.

Insertional mutagenesis provided additional information on sequence requirements within the 0.8-kb stabilization region (Fig. 4). As expected, the addition of base pairs upstream of the *parD* gene had no effect on stabilization of ParD-mediated repression of P*parD*, since this site lies approximately 25 bp from the minimal defined region sufficient for the expression of the *parDE* operon (11, 15). Alterations at the 5′ end of the *parD* structural gene inactivated plasmid stabilization and repression of P*parD*, demonstrating the requirement of ParD protein. However, the addition of extra codons at the central *parD Bgl*I site (and to a more limited degree, alteration of codons by a 3-bp deletion at this location) had no effect on the stabilization capacity, while repression of P*parD* was destroyed, suggesting that these properties represent two distinct functions of the ParD protein.

An interpretation of alterations at the 3′ end of the *parD* gene is somewhat more complicated. ParD-mediated repression of P*parD* does not depend on an intact ParD C terminus, since insertion of either 11 bp (which generates a ParD fusion that terminates eight codons past the *parE* start codon) or translational stop codons have no effect on P*parD* repression. Plasmid stabilization capacity, in contrast, is reduced or eliminated by the addition of 11 bp or stop codons at the 3′ end of *parD*. While this may reflect a requirement for an intact C-terminal portion of ParD, loss of stabilization could also be the result of a disruption of *parE* expression, which appears to rely on translational coupling with *parD* (36). The latter possibility is supported by the observation that stabilization capacity is partially restored when a chimeric ParD-ParE fusion protein is generated by the addition of 43 bp, which results in the translational fusion between ParD and ParE proteins (Fig. 4).

Recently, Ebrel et al. have identified protein secondary-structure homology between ParD and the class of DNA-binding proteins represented by the P22 proteins Arc and Mnt (15). These proteins function to control the transcription of the *ant* gene by binding within the promoter sequences (41, 42). Indeed, ParD has been shown to bind to DNA in the region of the P*parD* promoter (11, 15). However, unlike Arc and Mnt, the ParD protein must also play a role in plasmid stabilization, most likely employing properties separate from those required for the repression of P*parD*. The occurrence of mutants that cannot repress P*parD* but retain stabilization capacity suggest that DNA binding at the *parD* promoter is not essential for stabilization. It is possible that stabilization relies on the interaction of ParD and ParE, as suggested by the observation that while ParE is very basic, ParD is acidic (predicted pI of 4.8), representing potential charge-based pairing between the two.

It is of interest to consider the functional relationship between the 3.2- and 0.8-kb stabilization regions. It is clear that the *parCBA* operon encodes a multimer resolution system in which the ParA protein is most likely the resolvase acting within the *parCBA* and *parDE* operon promoter region (18, 21, 31). However, this region contributes functions to plasmid stabilization beyond multimer resolution, since resolution of multimers by the ColE1 *cer* site cannot functionally replace the *parCBA* operon (Table 5). The ParA, ParB, and/or ParC proteins may serve in an unknown manner to extend the ability of the *parDE* operon to stabilize plasmids, perhaps through the formation of a complex with ParD and/or ParE. These interactions may be essential for the maintenance of plasmid RK2 in a wide range of bacteria and under a variety of growth conditions.

ACKNOWLEDGMENTS

We thank David Summers for providing the constructs and strains for testing the ColE1 *cer* site, Doug Smith for providing the *himD* and *himA* strains, and Stanley Cohen for providing several of the *gyrA*, *gyrB*, and *topA* strains. We also thank Barbara Kittell, Aresa Toukdarian, and Arne Ström for critically reading this manuscript and Helmut Schwab for communicating his nucleotide sequence of the *parE* gene before its publication.

This work was supported by California Competitive Technology grant C90-078 and NIH grant AIO7194-26.

REFERENCES

1. **Appleyard, R. K.** 1954. Segregation of new lysogenic types during growth of a doubly lysogenic strain derived from *Escherichia coli* K12. Genetics **39**:440–452.
2. **Austin, S. J.** 1988. Plasmid partition. Plasmid **20**:1–9.
3. **Belasco, J. G., and C. F. Higgins.** 1988. Mechanisms of mRNA decay in bacteria: a perspective. Gene **72**:15–23.
4. **Biek, D. P., and S. N. Cohen.** 1989. Involvement of integration host factor (IHF) in the maintenance of plasmid pSC101 in *E. coli*: mutations in the *topA* gene allow pSC101 replication in the absence of IHF. J. Bacteriol. **171**:2066–2074.
5. **Bishop, P. E., and W. J. Brill.** 1977. Genetic analysis of *Azotobacter vinelandii* mutant strains unable to fix nitrogen. J. Bacteriol. **130**:954–956.
6. **Boyer, H. W., and D. Roulland-Dussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. **41**:459–472.
7. **Burkhardt, H., G. Riess, and A. Pühler.** 1979. Relationships of group P1 plasmids revealed by heteroduplex experiments: RP1, RP4, R68 and RK2 are identical. J. Gen. Microbiol. **114**:341–348.
8. **Carter, P., H. Bedouelle, and G. Winter.** 1985. Improved oligonucleotide site-directed mutagenesis using M13 vectors. Nucleic Acids Res. **13**:4431–4443.
9. **Casadaban, M. J., J. Chou, and S. N. Cohen.** 1980. In vitro gene fusions that join an enzymatically active β-galactosidase segment to amino-terminal fragments of exogenous proteins: *Escherichia coli* plasmid vectors for the detection and cloning of translational initiation signals. J. Bacteriol. **143**:971–980.
10. **Casadaban, M. J., and S. N. Cohen.** 1980. Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. J. Mol. Biol. **138**:179–207.
11. **Davis, T. D., D. R. Helinski, and R. C. Roberts.** 1992. Transcrip-

tion and autoregulation of the stabilizing functions of broad host range plasmid RK2 in *Escherichia coli*, *Agrobacterium tumefaciens* and *Pseudomonas aeruginosa*. Mol. Microbiol. **6**:1981–1994.

12. **Dellis, S., and M. Filutowicz.** 1991. Integration host factor of *Escherichia coli* reverses the inhibition of R6K plasmid replication by π initiator protein. J. Bacteriol. **173**:1279–1286.
13. **Ditta, G., S. Stanfield, D. Corbin, and D. R. Helinski.** 1980. Broad host range DNA cloning system for gram negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA **77**:7347–7351.
14. **Durland, R. H., and D. R. Helinski.** 1990. Replication of the broad-host-range plasmid RK2: direct measurement of intracellular concentrations of the essential TrfA replication proteins and their effect on plasmid copy number. J. Bacteriol. **172**:3849–3858.
15. **Eberl, L., M. Givskov, and H. Schwab.** 1992. The divergent promoters mediating transcription of the *par* locus of plasmid RP4 are subject to autoregulation. Mol. Microbiol. **6**:1969–1979.
16. **Funnell, B. E.** 1988. Participation of Escherichia coli integration host factor in the P1 plasmid partition system. Proc. Natl. Acad. Sci. USA **85**:6657–6661.
17. **Garfinkel, D. J., R. B. Simpson, L. W. Ream, F. F. White, M. P. Gordon, and E. W. Nester.** 1981. Genetic analysis of crown gall: fine structure map of the T-DNA by site-directed mutagenesis. Cell **27**:143–153.
18. **Gerlitz, M., O. Hrabak, and H. Schwab.** 1990. Partitioning of broad-host-range plasmid RP4 is based on a complex function including a site specific recombination system. J. Bacteriol. **172**:6194–6203.
19. **Granston, A. E., D. M. Alessi, L. J. Eades, and D. I. Friedman.** 1988. A point mutation in the *Nu1* gene of bacteriophage lambda facilitates phage growth in *Escherichia coli* with *himA* and *gyrB* mutations. Mol. Gen. Genet. **212**:149–156.
20. **Greener, A., S. M. Lehman, and D. R. Helinski.** 1992. Promoters of the broad host range plasmid RK2: analysis of transcription (initiation) in five species of Gram-negative bacteria. Genetics **130**:27–36.
21. **Grinter, N. J., G. Brewster, and P. T. Barth.** 1989. Two mechanisms necessary for the stable inheritance of plasmid RP4. Plasmid **22**:203–214.
22. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166**:557–580.
23. **James, A. A., P. T. Morrison, and R. Kolodner.** 1982. Genetic recombination of bacterial plasmid DNA. Analysis of the effect of recombination-deficient mutations on plasmid recombination. J. Mol. Biol. **160**:411–430.
24. **Kleiner, D., W. Paul, and M. J. Merrick.** 1988. Construction of multicopy expression vectors for regulated overproduction of proteins in *Klebsiella pneumoniae* and other enteric bacteria. J. Gen. Microbiol. **134**:1779–1784.
25. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
26. **Miller, C. A., S. L. Beaucage, and S. N. Cohen.** 1990. Role of DNA superhelicity in partitioning of the pSC101 plasmid. Cell **62**:127–133.
27. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
28. **Newton, J. W., P. W. Wilson, and R. H. Burris.** 1953. Direct demonstration of ammonia as an intermediate in nitrogen fixation by *Azotobacter*. J. Biol. Chem. **204**:445–451.
29. **Nordström, K., and S. J. Austin.** 1989. Mechanisms that contribute to the stable segregation of plasmids. Annu. Rev. Genet. **23**:37–69.
30. **Orr, E., N. F. Fairweather, I. B. Holland, and R. H. Pritchard.** 1979. Isolation and characterization of a strain carrying a conditional lethal mutation in the *cou* gene of *Escherichia coli* K12. Mol. Gen. Genet. **177**:103–112.
31. **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.
32. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
33. **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.
34. **Schägger, H., and G. von Jagow.** 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal. Biochem. **166**:368–379.
35. **Schwab, H.** Personal communication.
36. **Ström, A., R. C. Roberts, and D. R. Helinski.** Unpublished data.
37. **Studier, F. W., and B. A. Moffatt.** 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J. Mol. Biol. **189**:113–130.
38. **Summers, D. K., and D. J. Sherratt.** 1988. Resolution of ColE1 dimers requires a DNA sequence implicated in the three-dimensional organization of the cer site. EMBO J. **7**:851–858.
39. **Tabor, S., and C. C. Richardson.** 1985. A bacteriophage T7 polymerase/promoter system for controlled expression of specific genes. Proc. Natl. Acad. Sci. USA **82**:1074–1078.
40. **Thomas, C. M., and D. R. 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 Ltd., London.
41. **Vershon, A. K., S. Liao, W. R. McClure, and R. T. Sauer.** 1987. Bacteriophage P22 Mnt repressor: DNA binding and effects on transcription *in vitro*. J. Mol. Biol. **195**:311–322.
42. **Vershon, A. K., S. Liao, W. R. McClure, and R. T. Sauer.** 1987. Interaction of the bacteriophage P22 Arc repressor with operator DNA. J. Mol. Biol. **195**:323–331.
43. **Weinstein, M., R. C. Roberts, and D. R. Helinski.** 1992. A region of the broad-host-range plasmid RK2 causes stable in planta inheritance of plasmids in *Rhizobium meliloti* cells isolated from alfalfa root nodules. J. Bacteriol. **174**:7486–7489.
44. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33**:103–119.