JOURNAL OF BACTERIOLOGY, Oct. 1985, p. 446–455
0021-9193/85/100446-10$02.00/0
Copyright © 1985, American Society for Microbiology
Vol. 164, No.1

Regions of Broad-Host-Range Plasmid RK2 Involved in Replication and Stable Maintenance in Nine Species of Gram-Negative Bacteria

THOMAS J. SCHMIDHAUSER AND DONALD R. HELINSKI*
Department of Biology, University of California, San Diego, La Jolla, California 92093

Received 22 April 1985/Accepted 18 July 1985

The replication and maintenance properties of the broad-host-range plasmid RK2 and its derivatives were examined in nine gram-negative bacterial species. Two regions of RK2, the origin of replication (*oriV*) and a segment that encodes for a replication protein (*trfA* Δ*kilD*, designated *trfA), are sufficient for replication in all nine species tested. However, stable maintenance of this minimal replicon (less than 0.3% loss per generation under nonselection conditions) is observed only in *Escherichia coli*, *Pseudomonas aeruginosa*, *Pseudomonas putida*, and *Azotobacter vinelandii*. Maintenance of this minimal replicon is unstable in *Rhizobium meliloti*, *Agrobacterium tumefaciens*, *Caulobacter crescentus*, *Acinetobacter calcoaceticus*, and *Rhodopseudomonas sphaeroides*. A maintenance function has been localized to a 3.1-kilobase (kb) region of RK2 encoding three previously described functions: *korA* (*trfB korB1 korD*), *incP1*-(II), and *korB*. The 3.1-kb maintenance region can increase or decrease the stability of maintenance of RK2 derivatives dependent on the host species and the presence or absence of the RK2 origin of conjugal transfer (*oriT*). In the case of *A. calcoaceticus*, stable maintenance requires an RK2 segment that includes the promoter and the *kilD* (*kilB1*) functions of the *trfA* operon in addition to the 3.1-kb maintenance region. The broad-host-range maintenance requirements of plasmid RK2, therefore, are encoded by multiple functions, and the requirement for one or more of these functions varies among gram-negative bacterial species.**

Natural plasmid isolates of gram-negative bacteria belonging to incompatability groups C, N, P, Q, and W display replication and maintenance proficiency in a diversity of bacterial species. Most natural gram-negative plasmid isolates, however, can be established only in their host of origin and in closely related species. The basis for broad- versus narrow-host-range plasmid maintenance is poorly understood. Studies of narrow-host-range replicons suggest that their inability to be maintained in a particular organism is due to a lack of replication or segregation proficiency rather than to an inability to be introduced into or express genetic markers in a distantly related host (15, 24, 28, 29).

The incompatibility group P-1 plasmid RK2, similiar or identical to RP1, RP4, R18, and R68 (5, 36), has been the focus of studies to determine the basis of IncP-1 plasmid maintenance in *E. coli*. Extensive deletion analysis of plasmid RK2 has identified two regions of the 56.4-kilobase (kb) RK2 replicon, a 700-base-pair (bp) origin of replication (*oriV*), and a *trans*-acting function, *trfA*, that are essential for RK2 replication in *E. coli* (10, 21, 40). The *trfA* function and at least one additional function designated *kilD-kilB1* (25, 33) are expressed by the 1.5-kb *trfA* operon (30, 34). *kilD* activity leads to replicon instability in the absence of a kill override function designated *korD-korB1* (25, 33). Therefore, mini-RK2 replicons containing the *oriV* region and the *trfA* operon require a third region of RK2, containing the *korD* function, designated *trfB* (40). A deletion derivative of the *trfA* region, *trfA* Δ*kilD*, designated the *trfA** region, expresses the *trfA* function but not the *kilD* function and obviates the requirement for the *trfB* region (37).

We have previously shown that a replicon carrying only *oriV* and *trfA** encodes stable replication and maintenance properties in *Escherichia coli* and in the distantly related gram-negative bacterium *Pseudomonas putida* (26). Schwab et al. have characterized a spontaneous deletion derivative of RP4, pHS40, consisting of a continuous segment from *oriV* through the *trfA* region, as capable of replication in a third gram-negative species, *Alcaligenes eutropus* (27). Southern blot analysis of a worldwide collection of IncP-1 plasmids indicates that the *trfA** and *oriV* regions are conserved among all IncP-1 plasmids (6). These results are consistent with the proposal that these regions provide the minimal amount of information for replication of RK2. However, the *kilD-korD* circuit and perhaps additional functions encoded by RK2 may be required for the stable maintenance of this replicon in a wide variety of gram-negative species. Figurski et al. have identified a novel set of functions within plasmid RK2, designated *kilA*, *kilB*, and *kilC*, each of which leads to *E. coli* host cell death in the absence of corresponding kill override functions *korA*, *korB*, and *korC*, respectively (12). Genetic analysis of the *korA* (subsequent studies indicated that the *trfB-korD-korB1* and *korA* functions are identical [3, 13, 35, 41]) and *korB* functions indicates that these functions are conserved within a worldwide collection of 10 IncP-1 group isolates (12). The *korA* and *korB* functions have been shown to control RK2 copy number in *E. coli* and have been postulated to play a role in the broad-host-range properties of RK2 (3, 12, 13, 38).

Ditta et al. established that the RK2 conjugal transfer system, when joined to a narrow-host-range replicon, e.g., ColE1, was capable of mobilizing mini-RK2 derivatives that carry the RK2 origin of conjugal transfer (9). Guiney and Yakobson localized the functional origin of transfer (*oriT*) within a 760-bp *Hae*II fragment (16). Thus, a mini-RK2 replicon containing this *oriT* segment can be introduced into virtually any gram-negative bacterial host. The development of a proficient system for the introduction of mini-RK2 replicons into diverse gram-negative bacterial species and the identification of the *trfA** and *oriV* regions as components of a minimal RK2 replicon led us to undertake an assessment of the plasmid regions required for broad-host-range maintenance of plasmid RK2. Our results show that

* Corresponding author.

TABLE 1. Bacterial strains used

Bacterial species	Growth medium[a]	Tetracycline concn (μg/ml) used for selection	Medium for selection of transconjugants[b]	Source
E. coli MC1029	L	25		M. Casadaban
A. vinelandii Av uw	C	10	Burk MM with Tc	G. Ditta
P. putida 2440	L	50	M9 MM with Tc	M. Bagdasarian
P. aeruginosa PAO1161	L	100	M9 MM with leucine and Tc	M. Bagdasarian
R. meliloti 102F34	YMB	3	R MM with Tc	G. Ditta
A. tumefaciens A136	YEP	1	MM with Tc	E. Nester
R. sphaeroides 2.4.1 and WS8	L	1	L with Tc and Nal	S. Kaplan
C. crescentus CB15 CM5000	PYE	1	PYE with Tc and Nal	R. Bender
A. calcoaceticus BD413	L	30	L with Tc and Rif	I. Crawford

[a] The compositions of the various media and antibiotic concentrations are described in Materials and Methods.
[b] Abbreviations: MM, minimal medium; Nal, nalidixic acid; Rif, rifampin; Tc, tetracycline; L, L broth.

the *trfA** and *oriV* regions are sufficient for replication in each of nine bacterial species tested. However, the stable maintenance requirements of RK2 are complex, involve multiple regions including the *korA* and *korB* functions, and vary among the different gram-negative bacterial species tested.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains used are listed in Table 1. The genotypes of the bacterial strains employed were *E. coli* MC1029, *araD139 (ara leu)7697 (lacZ)M15 galU galK* StrA *recA56*; *Pseudomonas aeruginosa* PAO1161, *leu-38 hsdR*; *P. putida* 2440, an *hsdR* derivative of strain mt-2; and *Acinetobacter calcoaceticus* BD413, Str Rif. *Agrobacterium tumefaciens* A136 is a Rif^r Nal^r Ti plasmid-cured derivative of wild-type strain C58. Nalidixic acid-resistant isolates of *Rhodopseudomonas* strains 2.4.1 and WS8 were isolated in this laboratory. Wild-type strains of *Azotobacter*, *Caulobacter*, and *Rhizobium* species were used. The sources and relevant properties of plasmids used in this study are listed in Table 2 and in the text.

Media and antibiotic selection. Media used for growth of each bacterial species are listed in Table 1 and consisted of L broth (10 g of tryptone, 5 g of yeast extract, 10 g of NaCl, and 10 mM Tris [pH 7.5] per liter); C medium (0.8 mM $MgSO_4 \cdot 7H_2O$, 1.7 mM NaCl, 0.25% yeast extract, 0.05% Casamino Acids, 1% mannitol); YMB medium (1 g of yeast extract, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of K_2HPO_4, 0.1 g of NaCl, and 10 g of mannitol per liter); YEP medium (1% peptone, 1% yeast extract, 0.5% NaCl); and PYE medium (2 g of peptone, 1 g of yeast extract, and 0.2 g of $MgSO_4 \cdot 7H_2O$ per liter). Media used for the selection of transconjugants and the concentration of tetracycline used to select for plasmid-encoded antibiotic resistance are listed in Table 1 and consisted of modified Burk medium (0.2 g of KH_2PO_4, 0.8 g of K_2HPO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.09 g of $CaCl_2 \cdot 2H_2O$, 1 ml of FeMo [1 mg of Fe and 0.1 mg of Mo per ml], and 20 g of sucrose per liter) and R min medium (5.8 g of Na_2HPO_4, 3.0 g of KH_2PO_4, 0.5 g of NaCl, 1 g of mannitol, 0.5 mg of biotin, 0.2 g of $MgSO_4 \cdot 7H_2O$, and 0.5 g of glutamate per liter). M9 (11) and *Agrobacterium* spp. (43) minimal media have been described previously. Agar (1.5%) was added for solid medium. Nalidixic acid (30 μg/ml) was used to select transconjugants in *Rhodopseudomonas* and *Caulobacter* species. Rifampin (150 μg/ml) was used to select transconjugants in *Acinetobacter* spp.

Isolation of plasmid DNA. Clone analysis DNA was isolated from *E. coli*, *P. putida*, *P. aeruginosa*, *Rhizobium meliloti*, *Caulobacter crescentus*, *Rhodopseudomonas sphaeroides*, *Azotobacter vinelandii*, and *Acinetobacter calcoaceticus* by the alkaline sodium dodecyl sulfate method of Birnboim and Doly (4). Clone analysis DNA was isolated from *Agrobacterium tumefaciens* by the method of Corbin et al. (7). Purified plasmid DNA was prepared from *E. coli* by the cesium chloride-ethidium bromide equilibrium centrifugation method of Meyer et al. (20).

P. aeruginosa and *Rhodopseudomonas sphaeroides* were transformed with purified plasmid DNA by published methods (14, 39).

Conjugal matings. Patch matings were performed by mixing loopfuls of donor and recipient cells on the nonselective agar medium used for growth of the recipient (Table 1) for approximately 16 h at 30°C. Plasmid pRK2013 was used to mobilize transfer-defective, mobilization-proficient derivatives of RK2 (9). Transconjugants were selected by streaking the mating mixture onto an appropriate selective medium (Table 1). Transconjugants were tested for the presence of plasmid by restriction analysis of clone analysis DNA.

Assessment of plasmid maintenance. Plasmid-containing cultures generally were grown overnight at 30°C (all *E. coli* cultures were grown at 37°C) in growth medium supplemented with tetracycline for selection of plasmid-containing cells (Table 1). Cultures were then diluted 1:100 into 5 mls of fresh growth medium supplemented with tetracycline for 2 to 4 h at 30°C. Each culture was then diluted into 5 mls of nonselective growth medium prewarmed to 30°C to yield approximately 10^3 cells per ml. The 5-ml cultures were incubated with shaking on a New Brunswick Gyrotory bath. Cultures were grown to a concentration of between 5×10^5 and 5×10^8 cells per ml and then serially diluted into 5 mls of prewarmed growth medium to yield approximately 10^3 cells per ml, with the process repeated as necessary to complete the number of generations desired. After each dilution into fresh growth medium samples were taken and plated directly onto nonselective growth medium. The centers of single colonies were picked from these plates onto growth medium supplemented with tetracycline to assess the presence of plasmid-encoded antibiotic resistance. One hundred colonies were assayed for the presence of the plasmid-encoded antibiotic resistance at each subculturing.

RESULTS

Host range properties of RK2 and of a *trfA *oriV* replicon.** Plasmids pTJS75 and pTJS124 carry the *trfA**, Tc^r, and *oriV* regions of RK2 (Fig. 1 and 2). pTJS75 and pTJS124 also

TABLE 2. Plasmids used

Plasmid	Relevant genotype[a]	Reference or source
pCT88	Cm^r Tc^r, P15A replicon	37
pUC7, pUC8, pUC9	Ap^r, ColE1 replicon	42
pUC9CM	Cm^r Ap^s, derivative of pUC9	K. Buckley
pRK214.1	Tc^r Ap^r, 44.1-kb RK2 replicon	11
pRK215.1	Tc^r Ap^r, 49.5-kb RK2 replicon	11
pRK229	Tc^r, 24-kb RK2 replicon	40
pRK248	Tc^r, 9.6-kb RK2 replicon	40
pRK252	Tc^r, 10.3-kb RK2 replicon	8
pRK290	Tc^r, 20-kb RK2 replicon	9
pRK310	Tc^r, 20.4-kb RK2 replicon with the *Hae*II α-lac region of pUC9	8
pRK407	*Hae*II α-lac region of pUC9 ligated into *Hae*II site between *trfA* and *trfB* regions of plasmid pRK252	G. Ditta
pRK2178	Km^r, 6.8-kb P15A replicon with the kilobase 52.5 to 55.6 region of RK2	D. Figurski
pDG4	Ap^r, ColE1 replicon with the kilobase 45.0 to 56.4 region of RK2	16
pDG5	Ap^r, ColE1 replicon with the RK2 *Hae*II *oriT* region	16
pTJS26	Tc^r, 8.0-kb RK2 replicon	26
pTJS29A	Tc^r, 8.0-kb ColE1 replicon	26
pTJS50	Tc^r, 11.1-kb RK2 replicon	26
pTJS53	RK2 760-bp *Hae*II *oriT* region ligated as a blunt-end fragment into the *Bam*HI site of pUC8	This work
pTJS65	RK2 700-bp *Hae*II *oriV* region ligated as a blunt-end fragment into the *Sma*I site of pUC8	This work
pTJS66	RK2 700-bp *Hae*II *oriV* region ligated as a blunt-end fragment into the *Pst*I site of pTJS53	This work
pTJS67[b]	Tc^r, 9.8-kb RK2 replicon	This work
pTJS75[b]	Tc^r, 7.0-kb RK2 replicon	This work
pTJS81	RK2 760-bp *oriT* region ligated as a blunt-end fragment into *Pst*I-cut pUC7 (treatment with the Klenow fragment of DNA polymerase I removed all of the pUC7 multicloning site except for a single *Eco*RI site on either side of the *oriT* insert)	This work
pTJS87[b]	Tc^r, 11.4-kb RK2 replicon	This work
pTJS94	Tc^r, 8.8-kb RK2, P15A joint replicon; ligation of *Eco*RI-*Pst*I *oriV* of pTJS65 to the large *Eco*RI-*Pst*I fragment of pCT88	This work
pTJS100[b]	Tc^r, 19.5-kb RK2 replicon	This work
pTJS108	*Eco*RI Ap^r fragment derived from pBR322 ligated into the *Eco*RI site of pUC9CM	This work
pTJS111[b]	Tc^r, 14-kb RK2 replicon	This work
pTJS112[b]	Tc^r, 14.8-kb RK2 replicon	This work
pTJS117[b]	Ap^r Tc^r, 9.2-kb RK2 replicon	This work
pTJS124[b]	Ap^r Tc^r, 10-kb RK2 replicon	This work
pTJS129	Ligation of the 3.1-kb *Hinc*II fragment containing *korA*, *IncP1*-(II), and *korB* from plasmid pRK2178 into the *Hinc*II site of pUC9CM	This work
pTJS130[b]	Tc^r, 10.2-kb RK2 replicon	This work
pTJS133[b]	Tc^r, 11.0-kb RK2 replicon	This work
pTJS175[b]	Tc^r, 17.0-kb RK2 replicon	This work
pTJS194	Ligation of the *Hind*III Ap^r fragment of pTJS108 into the unique *Hind*III site of pTJS129 in the orientation yielding the 3.1-kb *korA–incP1*-(II)–*korB* region and Ap^r as an *Eco*RI fragment	This work
pTJS210[b]	Ap^r Tc^r, 13.0-kb RK2 replicon	This work

[a] Abbreviations: Cm, chloramphenicol; Tc, tetracycline; Ap, ampicillin; Km, kanamycin sulfate.
[b] Constructions described in the text.

contain the RK2 origin of transfer, *oriT*, as a 760-bp fragment that is sufficient to permit the mobilization of the plasmids into another bacterial host. Nine bacterial species were chosen as a representation of biologically important gram-negative species known to support IncP-1 group plasmids (Table 1). Our initial objective was to compare replication and maintenance properties of *trfA* oriV* minimal plasmid replicons to the parental RK2 plasmid in each host. Plasmid pRK290 (Fig. 1) was included in studies in each host as an intermediate size (20-kb) RK2 replicon known to have retained most of the host range of plasmid RK2 (8, 9). Plasmids pTJS75, pTJS124, pRK290, and RK2 were successfully introduced into each host tested, and all of the plasmids could be maintained by selection with the appropriate antibiotic. Thus, the minimal replication system consisting of the *oriV* and *trfA** regions is capable of replication in all nine of the bacterial hosts examined. Stable maintenance of the plasmids under conditions of bacterial growth without antibiotic selection was then tested as described in Materials and Methods. For comparative purposes in this study, stable maintenance is defined as less than 0.3% loss of the plasmid per generation. The results are summarized in Table 3. The intact 56.4-kb RK2 replicon displayed stable maintenance in all nine hosts tested. Plasmid pRK290 was maintained with various degrees of stability in all nine hosts tested. The rate of loss of pRK290 never exceeded 0.3% per generation in any host. While capable of replication in all nine hosts tested, the *trfA* oriV* replicons pTJS75 and pTJS124 lacked the ability to be stably maintained in six of the nine hosts tested, i.e., in *P. aeruginosa*, *Rhodopseudomonas sphaeroides*, *Caulobacter crescentus*, *A. calcoaceticus*, *Rhizobium meliloti*, and *A. tumefaciens*. In two hosts, *R. meliloti* and *A. tumefaciens*, plasmid pTJS124 exhibited significantly greater stability then did plasmid pTJS75.

Host range properties of the *trfA* operon, *korA*, and *IncP1*-(II) functions of RK2. The relative instability of plasmids pTJS75 and pTJS124 in six of the nine hosts tested suggests a requirement for functions in addition to the *trfA** and *oriV* regions for RK2 broad-host-range maintenance. Analysis of the *trfA* region has shown that a single promoter expresses

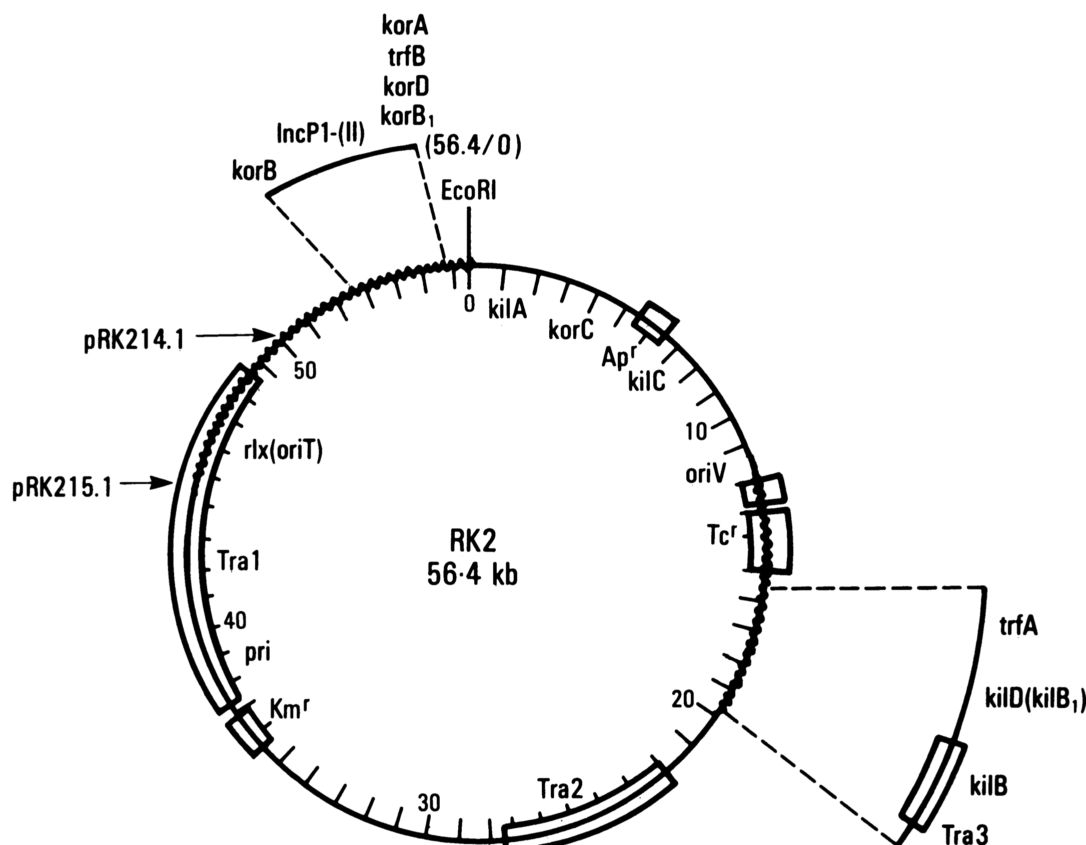

FIG. 1. Genetic map of RK2 incorporating information from RP1 and RP4, similar or identical to RK2 (5). The RK2 genome is calibrated in kilobase-pair coordinates from the unique *Eco*RI site. Two regions of RK2 involved in broad-host-range replication and maintenance have been expanded for clarity. *tra* refers to conjugal transfer functions, *rlx* refers to the relaxation complex (origin of conjugal transfer) site (16), and *pri* refers to a DNA primase (19). The balance of genetic loci shown are described in the text. Regions of RK2 present in pRK290 are indicated by a wavy line. The positions of the bacteriophage Mu insertions into RK2 which were used to construct plasmids pRK214.1 and pRK215.1 are shown by arrows (11).

three polypeptides of 43, 32, and 13 kilodaltons in *E. coli* (18, 30, 34, 41). In these studies it was proposed that the 32- and possibly the 43-kilodalton polypeptides encode the *trfA* function, while the 13-kilodalton polypeptide represents the *kilD-kilB1* function defined as destabilizing RK2 mini-replicon maintenance in the absence of the *korA* (*trfB-korD-korB1*) function. Thus, an intact *trfA* operon requires the *korA* function to suppress the *kilD* function. The *trfA** region was derived from the *trfA* region by a Tn*5* insertion and restriction endonuclease deletion (37). We have determined by DNA sequence analysis that the Tn*5* insertion creating the *trfA** region maps to base pair 34 in the *trfA* operon (Fig. 3; unpublished data). Thus, the *trfA** region lacks the *trfA* promoter and the first 4 bp of the *kilD* coding region as well. Presumably, in the *trfA** region the *trfA* function is expressed from an outside promoter. To test the ability of the intact *trfA* operon to support (along with *oriV*) stable RK2 broad-host-range maintenance requires the presence of the *korA* function to suppress the *kilD* function as outlined above. In addition to the RK2 origin of replication, a second region of RK2 has been found to determine IncP-1 group incompatibility. This function, termed *IncP1*-(II), maps adjacent to *korA* and may be expressed from the same promoter as the *korA* function (22, 35, 41).

We compared the maintenance properties of a mini-RK2 replicon consisting of the *trfA*, *korA*, *incP1*-(II), *oriV*, and *oriT* regions (plasmid pRK252, Fig. 4 and 5) to the properties of mini-RK2 plasmids containing the *trfA**, *korA*, *incP1*-(II), *oriV*, and *oriT* regions (pTJS67 or pTJS87, Fig. 2) in several bacterial hosts (Table 3). In each host tested, direct comparison of pTJS67, pTJS87, or pRK252 with pTJS75 or pTJS124 indicated similar or less proficient maintenance of the replicons containing the intact *trfA* region or the *korA*–*incP1*-(II) region or both. Thus, the intact *trfA* operon and *korA*–*incP1*-(II) regions are insufficient to provide maintenance functions to the minimal RK2 replicon in the bacterial hosts tested.

Localization of host range functions in plasmid pRK290. The 20 kb of RK2 DNA present in plasmid pRK290 appear to encode maintenance functions that are absent from the smaller deletion derivatives used in this study. Plasmid pRK290 consists of approximately 8 kb of RK2 DNA extending clockwise from *oriV* through the *kilB* region and including the *trfA* operon (kilobases 12.0 to 20.0 on the RK2 map) and 12 kb of RK2 extending from within the tra1 region clockwise to just beyond the single *Eco*RI site of RK2 (kilobases 45.0 to 0.3 on the RK2 map; Fig. 1). We attempted to identify sequences necessary for broad-host-range maintenance within each of these two regions of plasmid pRK290. The region of RK2 between kilobases 45.0 and 56.4 was joined to the *oriV*, Tc^r, and *trfA** regions of RK2 (coordinates, 12.0 to 17.6 kb) as plasmid pTJS100 (Fig. 4) or to the *oriV*, Tc^r, and *trfA* regions (coordinates, 12.0 to 18.7 kb) as plasmid pTJS175 (Fig. 4).

TABLE 3. Plasmid maintenance in various bacterial hosts[a]

Plasmid	Regions of RK2[b]	Avg % loss of tetracycline resistance[c] in:									
		E. coli (*n* = 50)	*Azotobacter* spp. (*n* = 35)	*P. putida* (*n* = 39–52)	*P. aeruginosa* (*n* = 40–45)	*R. meliloti* (*n* = 43–49)	*Agrobacterium* spp. (*n* = 34–36)	*R. sphaeroides* 2.4.1 (*n* = 25–29)	*R. sphaeroides* WS8 (*n* = 42–46)	*Caulobacter* spp. (*n* = 31–33)	*Acinetobacter* spp. (*n* = 27–32)
RK2		0	0	1	1	0	0	0	—	0	1
pRK290	Fig. 1	2	1	1	13[d]	1	11	—	3	7	11
pTJS75	*trfA**	0	0	9	55	66	43	68	—	—	97
pTJS124	*trfA**	—	—	—	—	19	18	53	76	90	—
pRK252	*trfA*, 54.1–56.4 kb	35	3	96	99[e]	49	88	86	—	99	90
pTJS67	*trfA**, 54.1–56.4 kb	1	0[f]	13	91	88	—	89	—	—	95
pTJS87	*trfA**, 54.1–56.4 kb	—	—	—	82	37[d]	52[d]	—	—	100	—
pTJS100	*trfA**, 45.0–56.4 kb	0	[g]	—	—	3	—	5	—	14	32
pTJS175	*trfA*, 45.0–56.4 kb	9	—	100[e]	—	19	—	—	10	—	36
pTJS112	*trfA**, 50.4–56.4 kb	1	[g]	9	—	0	—	0	—	3	76[d]
pTJS133	*trfA**, 52.5–55.6 kb	0	[g]	98[e]	3	0	4	3	0	2	88
pTJS210	*trfA*, 52.5–55.6 kb	1	—	41	44	10	61	—	8[d]	20	0
pTJS26	*trfA**, no *oriT*	1	—	10	9	—	—	—	36	—	—
pTJS50	pTJS87 without *oriT*	0	—	8	22	—	—	—	—	—	—
pTJS130	pTJS133 without *oriT*	0	—	[g]	—	—	—	—	58	—	—
pTJS91	Fig. 5	0	—	4	—	2	2	—	74	74	18
pTJS95	Fig. 5	3	—	93[d]	48	2	14	—	2[d]	5	64[d]

[a] Bacterial species are listed in Table 1.
[b] All plasmids contain the RK2 700-bp *oriV* region. Unless otherwise indicated, each plasmid also contains the RK2 760-bp *oriT* region. Other regions relevant to maintenance properties are indicated.
[c] Loss of resistance from cultures grown in the absence of selection for tetracycline resistance for *n* generations as described in Materials and Methods. Each value represents the average of three or more studies unless othewise indicated. —, Plasmid maintenance was not assessed. The *trfA* and *oriT* regions and the coordinate system used are indicated in Fig. 1. The *trfA** region is shown in Fig. 3.
[d] Average of two studies.
[e] Plasmid-encoded tetracycline resistance was lost from more than 90% of the cells approximately 15 generations earlier than the final value for *n*.
[f] Result of a single study.
[g] Plasmid could not be introduced into the host in three or more conjugal mating attempts or transformations with purified DNA.

Comparison of pTJS175 to pRK252 in five hosts indicates that the region of RK2 between kilobases 45.0 and 56.4 encodes a function(s) that increases the stability of maintenance of the minimal RK2 replicon in *E. coli*, *R. meliloti*, *R. sphaeroides*, and *A. calcoaceticus* (Table 3). However, both pRK252 and pTJS175 are unstable in *P. putida*. Plasmid pTJS100 exhibits stable maintenance in *R. meliloti*, *R. sphaeroides*, and *C. crescentus*, yet is relatively unstable in *A. calcoaceticus* and could not be established in *Azotobacter vinelandii* (Table 3). The instability of pTJS100 and pTJS175 in *A. calcoaceticus* suggests that although the region of RK2 between kilobases 45.0 and 56.4 contributes to RK2 maintenance in this host (comparison of pTJS100 to pTJS75 and of pTJS175 to pRK252), the *kilB* function or adjacent sequences present in pRK290 but not in pTJS100 or pTJS175 may be important in RK2 maintenance in *A. calcoaceticus*.

Identification of a 3.1-kb host range maintenance region. To localize the maintenance function(s) encoded by the segment of RK2 between kilobases 45.0 and 56.4, two derivatives of this region were examined. The region of RK2 between kilobases 50.4 and 56.4, with 1 kb from the left end of phage Mu, was joined to the *oriV*, *trfA**, and *oriT* regions of RK2 as plasmid pTJS112 (Fig. 4). Maintenance properties of pTJS112 in *R. meliloti*, *R. sphaeroides*, and *C. crescentus* suggest that plasmid pTJS112 contains all the host range maintenance functions present within pTJS100 (Table 3). In *A. calcoaceticus*, plasmid pTJS112 is significantly less stable than is pTJS100, indicating that additional maintenance functions may be present in the approximately 5.4 kb of DNA present in pTJS100 but not in pTJS112 (the region of RK2 between kilobases 45.0 and 50.4).

Our studies with plasmids pTJS67, pTJS87, and pRK252 indicate that the 2.3-kb *korA*–*incP1*-(II) region (RK2 coordinates, 54.1 to 56.4 kb) is insufficient to provide host range maintenance function(s) to a *trfA oriV* or *trfA** *oriV* replicon. The *korA* and *korB* functions of RK2 have been localized to a 3.1-kb *Hin*cII fragment (the region of RK2 between kilobases 52.5 and 55.6; D. H. Bechhofer and D. H. Figurski, unpublished data). Joining of the 3.1-kb region containing the *korA*, *incP1*-(II), and *korB* functions to the *oriV*, *trfA**, and *oriT* regions of RK2 yields plasmid pTJS133 (Fig. 4). Joining of the *oriV*, *trfA*, and *oriT* regions of RK2 to the region between kilobases 52.5 and 55.6 yields plasmid pTJS210 (Fig. 4). pTJS133 is stable in six of nine hosts tested. pTJS210 is stable in four of eight hosts tested (Table 3). Thus, the region between kilobases 52.5 and 55.6 but not that between 54.1 to 56.4 er ›des RK2 broad-host-range maintenance function(s) when joined to a *trfA** *oriV* or a *trfA*

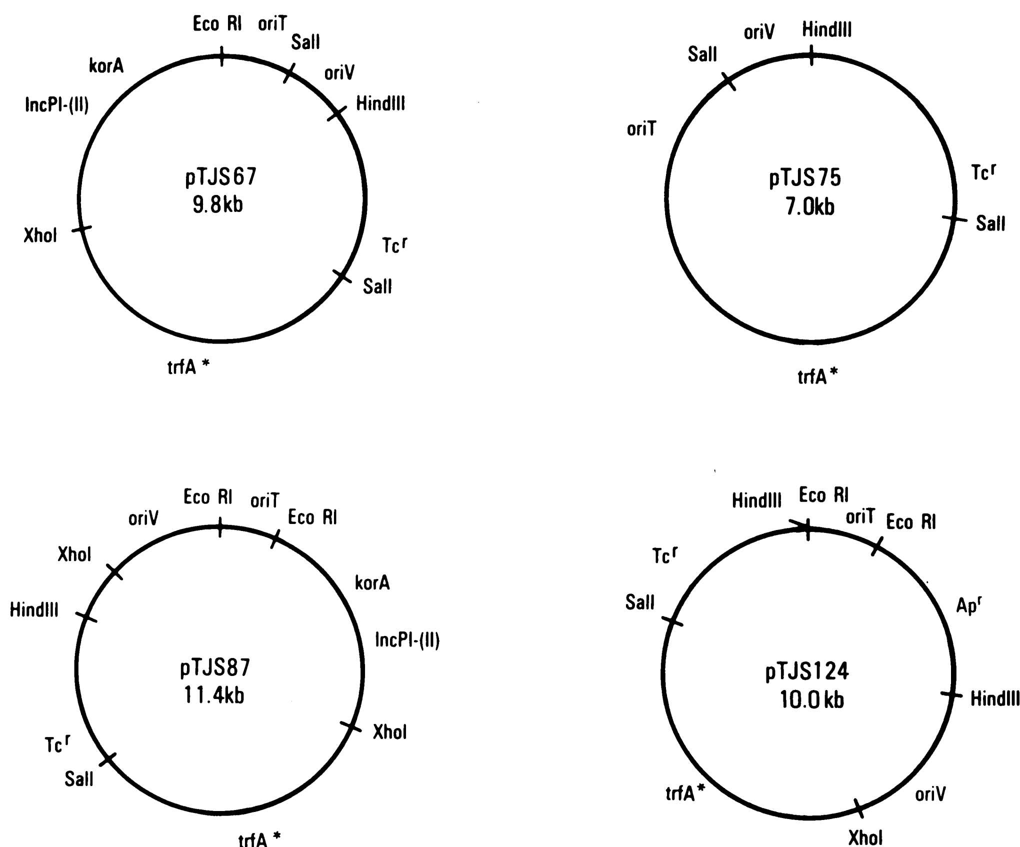

FIG. 2. Structure and relevant genetic loci and nuclease cleavage sites of plasmids pTJS67, pTJS75, pTJS87, and pTJS124. These plasmids contain the *trfA**, *oriV*, and *oriT* regions with and without the *korA–incP1*-(II) region. pTJS67 was constructed by ligation of the large *Hin*dIII-*Eco*RI fragment of pTJS29A to the small *Hin*dIII-*Eco*RI fragment of pTJS66. pTJS67 was digested with *Eco*RI and *Xho*I, treated with DNA polymerase I Klenow fragment to produce blunt ends, and ligated to yield plasmid pTJS75. Plasmid pTJS87 is the product of insertion of the *Eco*RI RK2 *oriT* fragment of pTJS81 into the unique *Eco*RI site of pTJS50. The *Hin*dIII Ap^r fragment of pTJS108 was ligated to *Hin*dIII-cleaved pTJS26 to yield plasmid pTJS117. Plasmid pTJS124 is the product of insertion of the *Eco*RI RK2 *oriT* fragment of pTJS81 into the unique *Eco*RI site of pTJS117.

oriV replicon. However, the 3.1-kb *korA–incP1*-(II)–*korB* region appears to destabilize a *trfA** region *oriV* replicon in *P. putida* and in *A. vinelandii* (comparison of pTJS75 and pTJS133).

The stable maintenance of plasmid pTJS210 in *A. calcoaceticus* and the instability of every *trfA** region replicon tested in this host indicates that the *kilD* function or the *trfA* operon promoter present in pTJS210 but not in *trfA** region plasmids, or both, are essential for RK2 maintenance in this host. Conversely, the stable maintenance of the *trfA** region plasmids pTJS75 or pTJS133 in every host tested save *A. calcoaceticus* indicates that the *trfA* operon promoter and the intact *kilD* coding region are not essential for RK2 mini-replicon maintenance in many of the gram-negative bacterial species.

Influence of the RK2 *oriT* region on plasmid broad-host-range maintenance. In a previous study, the *trfA** *oriV* plasmid pTJS26 exhibited stable maintenance properties in *E. coli* and *P. putida* (26). Plasmid pTJS26 was introduced into *P. aeruginosa* PAO1161 via transformation (see Materials and Methods). Maintenance properties of pTJS26 in strain PAO1161 indicate that a *trfA** *oriV* replicon can be stably maintained in this host (Table 3). Comparison of pTJS26 with pTJS75 in strain PAO1161 indicates that the presence of the RK2 *oriT* in pTJS75 leads to replicon instability in *P. aeruginosa*. Plasmid pTJS87 was derived from pTJS50 by the insertion of RK2 *oriT* as a 760-bp *Eco*RI fragment (Fig. 2). Comparison of pTJS50 and pTJS87 with pTJS133 in strain PAO1161 confirms the observation that the 760-bp RK2 *oriT* segment confers instability in *P. aeruginosa* in the absence of the *korA–incP1*-(II)–*korB* region (Table 3).

These observations on the RK2 *oriT* region were extended to *R. sphaeroides*. In this bacterium, mini-RK2 plasmids with RK2 *oriT* are unstable regardless of whether replication is dependent on the *trfA* and *oriV* regions or on the *trfA** and *oriV* regions unless the region between kilobases 52.5 and 55.6 is also present (Table 3; comparison of pTJS75, pTJS124, pRK252, pTJS133, and pTJS210). To further test this effect of *oriT*, *R. sphaeroides* WS8 Nal was transformed with purified pTJS26 DNA (see Materials and Methods). Comparison of the maintenance properties of plasmids pTJS26, pTJS124, and pTJS133 in strain WS8 Nal indicates that the RK2 *oriT* region contributes to replicon instability in this host and that the region between kilobases 52.5 and 55.6 can stabilize replicons containing RK2 *oriT* in strain WS8 Nal (Table 3). The complete stability of pTJS133 in strain WS8 Nal suggests that the *korA–incP1*-(II)–*korB* region can stabilize RK2 *oriT*-containing replicons and provide maintenance functions absent in the *trfA** *oriV* plasmid pTJS26 in this host.

Destabilization of mini-RK2 plasmids by the 3.1-kb

***korA-incP1*-(II)-*korB* region.** The finding that the region of RK2 between kilobases 52.5 and 55.6 appears to counteract instability originating in the RK2 *oriT* region in at least two hosts (*P. aeruginosa* and *R. sphaeroides*) suggests an interaction between these two regions of RK2. To investigate this relationship, the maintenance properties of pTJS130 were examined. pTJS130 is the precursor to plasmid pTJS133 and consists of the *oriV*, *trfA**, and kilobases 52.5 to 55.6 regions of RK2 (Table 1). Comparison of the maintenance properties of pTJS130 and pTJS133 in strain WS8 Nal indicates that the absence of RK2 *oriT* in plasmid pTJS130 leads to an unstable replicon in this host (Table 3). Repeated attempts to introduce plasmid pTJS130 into *P. putida* by transformation were unsuccessful, suggesting that the plasmid is extremely unstable in this host. Thus, in two gram-negative bacteria where the kilobase 52.5 to 55.6 region of RK2 counteracts instability due to the presence of the RK2 *oriT* region, the kilobase 52.5 to 55.6 region of RK2 appears to lead to unstable plasmid maintenance in the absence of RK2 *oriT*. Furthermore, in *P. putida*, pTJS133 is very unstable relative to *trfA** *oriV oriT* replicons which lack the *korB* region (e.g., pTJS75 or pTJS67). Finally, repeated attempts to introduce *trfA** *oriV* replicons which contain the kilobase 52.5 to 55.6 region of RK2 (pTJS100, pTJS112, and pTJS133) into *A. vinelandii* were unsuccessful. These results suggest that although the region between kilobases 52.5 and 55.6 is important in RK2 broad-host-range maintenance, the interaction of this region with other functions within RK2 such as in the *oriT* region may also play a role in stable maintenance in specific gram-negative bacteria.

The RK2 *korA* function has been shown to suppress the *kilA* (12, 13) and *kilD* (*kilB1*) functions (25) and to allow expression of the *korB* and *korC* functions (44). The studies of Smith and Thomas (33, 35) have also demonstrated that *korA* (*trfB*) is necessary to suppress *kilD*. Shingler and Thomas have shown that the *korA* function regulates expression of the *trfA* promoter (30). In addition, the *korB* function (which depends on the *korA* function for expression) regulates expression of the *trfA* promoter and the putative *kilB* promoter (30, 41).

Effect on host range of the *kilA*, *kilC*, and *korC* functions. To test the importance of the complete *kil-kor* circuits, two additional plasmids were constructed and examined for stable maintenance. Plasmid pRK229 has been shown to contain the complete *kil-kor* system (33). The addition of RK2 *oriT* to plasmid pRK229 yields plasmid pTJS91 (Fig. 5). The *kilA*, *korC*, *kilC*, *oriV*, and Ap^r regions of pRK229 were replaced with the RK2 *oriV* and *oriT* regions of plasmid pRK252 to yield plasmid pTJS95 (Fig. 5). Plasmids pTJS91 and pTJS95 are stably maintained in *E. coli*, *R. meliloti*, and *A. tumefaciens*. In two hosts, *R. sphaeroides* and *C. crescentus*, pTJS95 is stable, while pTJS91 maintenance is unstable. In two other hosts, *P. putida* and *A. calcoaceticus*, pTJS91 is stable while pTJS95 is unstably maintained (Table 3). Thus, as was observed in our studies of the region between kilobases 52.5 and 55.6, functions present in pTJS91 or pTJS95 which contribute to increased stability of plasmid maintenance in one host may create instability in another host.

DISCUSSION

We have examined the maintenance properties of derivatives of the IncP-1 plasmid RK2 to determine the genetic basis of broad-host-range plasmid maintenance. Stable RK2 maintenance was observed in a representation of gram-negative bacteria confirming the broad-host-range properties

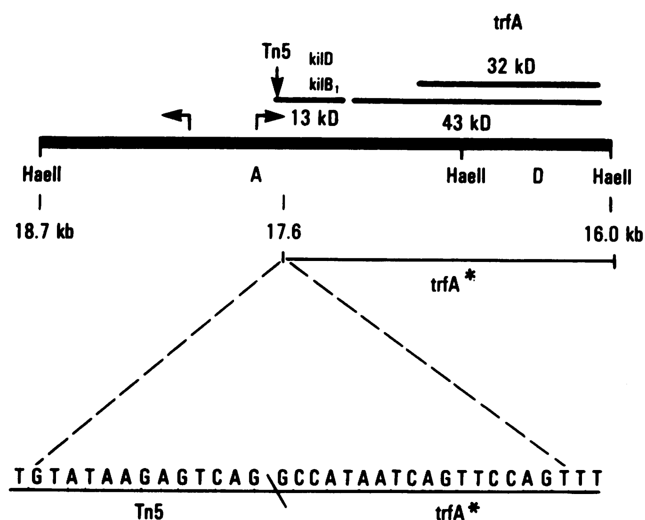

FIG. 3. The *Hae*II fragments of the *trfA* region are depicted after Smith et al. (32). The leftward putative *kilB* promoter and rightward *trfA* promoter are indicated by arrows. The three polypeptides encoded by the *trfA* operon are shown (18, 30). An arrow indicates the site of the Tn*5* insertion used to construct the *trfA** derivative of the *trfA* region (37). Below the site of the *trfA** Tn*5* insertion, the sequence of the Tn*5*-*trfA* operon junction is shown. Comparison with the sequence of the intact *trfA* operon indicates that deletion of the balance of Tn*5* to create the *trfA** region has removed the *trfA* promoter and the first 4 bp of the *kilD* coding region (unpublished data). kD, Kilodaltons.

of this member of the IncP-1 group. Analysis of self-replicating deletion derivatives of RK2 in nine bacterial species has established that 2.2 kb of RK2 DNA containing the *trfA** and *oriV* regions encodes RK2 broad-host-range replication properties. However, this *trfA** *oriV* replicon does not contain sufficient information for broad-host-range maintenance. While stably maintained in *E. coli*, *P. putida*, *P. aeruginosa*, and *A. vinelandii*, the *trfA** *oriV* replicon (pTJS26, pTJS124, or pTJS75) is not stably maintained in *R. sphaeroides*, *C. crescentus*, or *A. calcoaceticus*. Furthermore, the presence of the RK2 *oriT* region increases instability of a *trfA** *oriV* plasmid (pTJS75 and pTJS124) in *P. aeruginosa* and *R. sphaeroides*.

DNA sequence analysis of the *trfA** derivative of the *trfA* region indicates that the *trfA* promoter and the first 4 bp of the *kilD* coding sequence have been replaced by the end of transposon Tn*5*. Stable maintenance of RK2 derivatives in *A. calcoaceticus* is dependent on the presence of the intact *trfA* operon. However, the *trfA* operon promoter and the intact *kilD* coding region were nonessential for stable RK2 maintenance in the other gram-negative bacterial species in our study. RK2 maintenance functions were localized to the kilobase 52.5 to 55.6 region of RK2. The addition of this 3.1-kb region to a *trfA oriV* plasmid (pTJS210) allows stable maintenance in four of the eight hosts tested. In addition, the region between kilobases 52.5 and 55.6 stabilized a *trfA** region *oriV* plasmid (pTJS133) in *R. meliloti*, *A. tumefaciens*, and *C. crescentus* (comparison with pTJS124) and reduced instability arising from the RK2 *oriT* region in *P. aeruginosa* and *R. sphaeroides* (comparison of pTJS26, pTJS75, pTJS124, and pTJS133). However, addition of the RK2 region between kilobases 52.5 and 55.6 destabilized a plasmid in certain situations (comparison of pTJS75 and pTJS133 in *P. putida* and *A. vinelandii* and of pTJS26 and pTJS130 in strain WS8 Nal). Thus, the functions present in

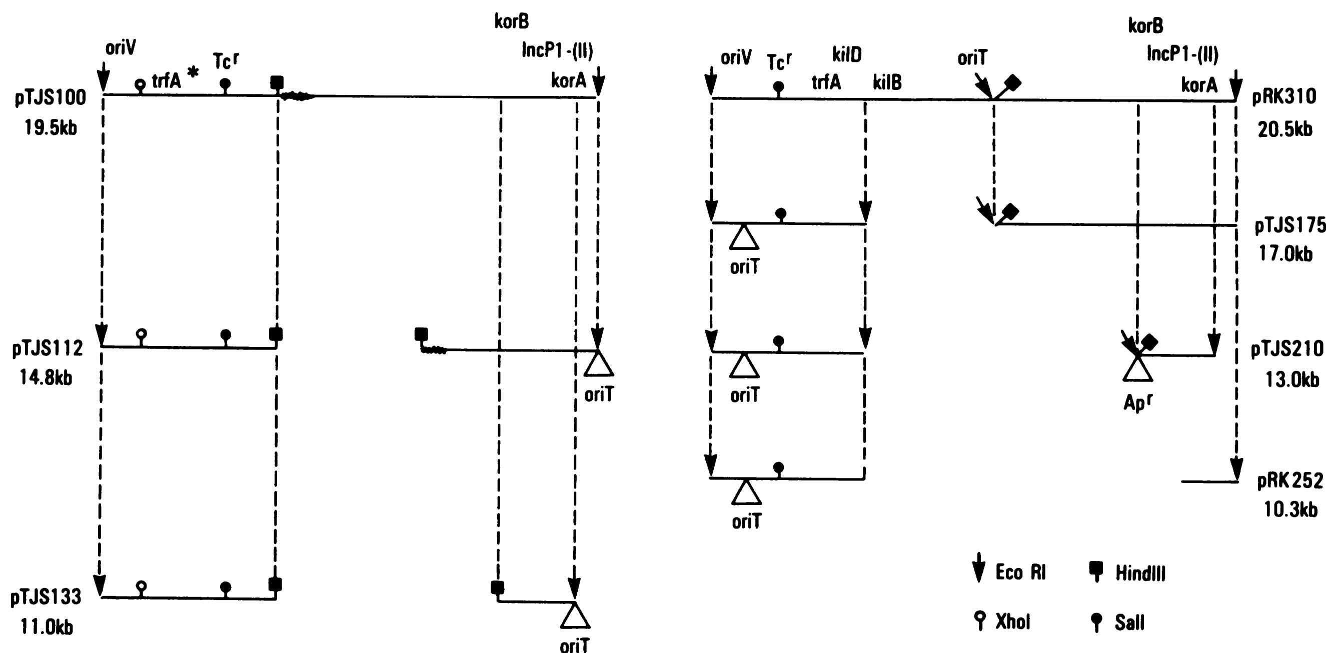

FIG. 4. Structural features and relevant genetic loci and nuclease digestion sites of RK2 derivatives. Plasmid pTJS100 consists of the large *Hin*dIII-*Eco*RI fragment of pTJS94 (containing the *oriV*, *trfA**, and Tc^r regions of RK2) joined to the small *Hin*dIII-*Eco*RI fragment of pRK215.1 (Fig. 1). Plasmid pRK310 consists of the α-lac *Hae*II fragment of pUC9 ligated into pRK290 linearized with *Hae*II (8). Plasmid pTJS111 was constructed by replacing the pRK215.1-derived *Hin*dIII-*Eco*RI fragment of pTJS100 with the small *Hin*dIII-*Eco*RI fragment of pRK214.1 (Fig. 1). Plasmid pTJS112 is the product of insertion of the *Eco*RI RK2 *oriT* fragment of pTJS81 into the unique *Eco*RI site of pTJS111. Mu sequences present in pTJS100 and pTJS112 are indicated by a wavy line. Plasmid pTJS130 was constructed by replacing the pRK215.1-derived *Hin*dIII-*Eco*RI fragment of pTJS100 with the 3.1-kb *Hin*dIII-*Eco*RI fragment of pTJS129. Plasmid pTJS133 is the product of insertion of the *Eco*RI RK2 *oriT* fragment of pTJS81 into the unique *Eco*RI site of pTJS130. Plasmid pTJS175 is the product of ligation of the small *Eco*RI fragment of pRK310 and the large *Eco*RI fragment of pRK407. Plasmid pTJS210 is the product of ligation of the large *Eco*RI fragment of pRK407 to an *Eco*RI fragment containing the ampicillin resistance gene, *korA*, *incP1*-(II), and *korB* from plasmid pTJS194.

the RK2 region between kilobases 52.5 and 55.6 appear to increase or decrease the stability of RK2 mini-replicon maintenance dependent on the bacterial host and the presence of the RK2 *oriT* region.

Relationship of host range maintenance functions to previously identified RK2 functions in the RK2 region between kilobases 52.5 and 55.6. The region of RK2 between kilobases 52.5 and 55.6 contains three previously described

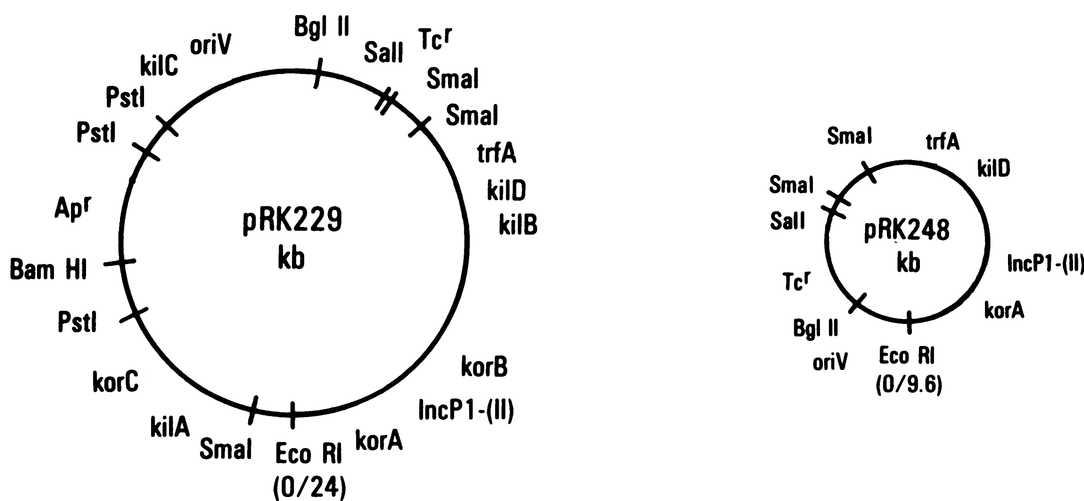

FIG. 5. Structure and relevant genetic loci and nuclease digestion sites of plasmids pRK229 and pRK248. Plasmid pTJS91 was derived from pRK229 by insertion of the *Eco*RI RK2 *oriT* fragment of pTJS81 into the unique *Eco*RI site of pRK229. Plasmid pRK252 was derived from plasmid pRK248 by insertion of the *Hae*II RK2 *oriT* of pDG5 between pRK248 coordinates 1.1 and 2.3 with the concurrent loss of the *Bgl*II site containing the *Hae*II fragment of pRK248 (8). Plasmid pTJS95 is the product of the ligation of the small *Eco*RI-*Sal*I fragments of pRK229 and pRK252.

functions, i.e., *korA*, *incP1*-(II), and *korB*. Preliminary results assessing Bal 31 deletions of the this region indicate that deletions resulting in either a $korA^-$ $korB^-$ phenotype (the *korA* function is required for *korB* expression [13]) or a $korA^+$ $korB^-$ phenotype are unable to provide host range maintenance functions to a *trfA** region *oriV* plasmid in *R. meliloti*, *P. aeruginosa*, or *R. sphaeroides* (T. J. Schmidhauser, D. H. Bechhofer, D. H. Figurski, and D. R. Helinski, unpublished data). We have also shown that the *korA–incP1*-(II) region (kilobases 54.1 to 56.4) in the absence of *korB* function cannot provide RK2 maintenance functions to a *trfA oriV* or a *trfA** region *oriV* plasmid (pRK252, pTJS67, or pTJS87). These results suggest that the entire *korA–incP1*-(II)–*korB* coding region is required to provide maintenance functions.

What is the genetic basis of the broad-host-range maintenance phenotype of the region between kilobases 52.5 and 55.6? Evidence has been obtained for the control of RK2 copy number by the *korA* and *korB* functions (38). It has been proposed that *korB* controls RK2 copy number by regulating the expression of the *trfA* promoter (38, 41). However, we have shown that the region between kilobases 52.5 and 55.6 provides maintenance function(s) to a mini-RK2 plasmid lacking the *trfA* promoter (pTJS133), suggesting that the *korB* function may control RK2 copy number by a mechanism other than or in addition to the regulation of the *trfA* promoter. The *korA* function appears to act as an anti-terminator of *korB* and *kilA* transcription (13, 44). However, this study has shown that the *korA* region alone does not encode a broad-host-range maintenance phenotype. It is possible that the *korA–incP1*-(II)–*korB* region functions as a broad-host-range partition, coupled cell division (17, 23), or copy number control locus able to act on mini-RK2 plasmids with and without the *trfA* promoter.

The results with our various plasmid constructs suggest the involvement of regions in addition to the *trfA* operon and the segment between kilobases 52.5 and 55.6 in broad-host-range maintenance. The 760-bp RK2 *oriT* region destabilizes *trfA** *oriV* plasmids in *P. aeruginosa* and *R. sphaeroides*. In addition, the region between kilobases 52.5 and 55.6 destabilizes a *trfA** *oriV* replicon (pTJS133) in *R. sphaeroides* and potentially in *P. aeruginosa* unless the RK2 *oriT* region is present. Comparison of the maintenance properties of plasmids pTJS133 and pTJS112 in *P. putida* suggests that the region of RK2 between kilobases 50.4 and 52.5 or the 1 kb of the left end of phage Mu present in plasmid pTJS112 allows stable maintenance in this host.

Barth et al. (1, 2) have shown that a Tn*76* insertion between the *korA* and *korB* functions of RP4 (plasmid pRP761) has a differential effect on RP4 maintenance dependent on the gram-negative host. Thomas et al. observed that a *trfA oriV* plasmid spontaneously mutated to reduce expression of the *kilD* function, while capable of replication in *E. coli*, could not be introduced into *P. aeruginosa* unless the *korA–incP1*-(II) region was provided in *cis* (39). These results suggest that the expression or phenotype or both of *kil-kor* functions may vary among gram-negative species. Alternatively or additionally, non-*kil-kor* functions may be responsible for the maintenance properties observed, and the structure of RK2 derivatives may influence their expression. We observed that two plasmids, pTJS67 and pTJS75, were unstable in two hosts, *R. meliloti* and *A. tumefaciens*, relative to two plasmids consisting of the same regions of RK2 joined in a different order (pTJS87 and pTJS124, respectively). Preliminary analysis suggests that the close proximity of the *oriV* and *oriT* regions of RK2 in plasmids pTJS67 and pTJS75 destabilizes the plasmids relative to pTJS87 and pTJS124 in *R. meliloti* and *A. tumefaciens*. Subsequently, the replicons in our study were constructed to minimize, but do not rule out, structural effects on the stability of replicon maintenance. Indeed, position effects, rather then the RK2 region between kilobases 52.5 and 55.6, appear to be responsible for the instability of pTJS133 in *Azotobacter* spp. and *P. putida* (unpublished observations).

Our own observations and those of other laboratories clearly indicate the interaction of several regions of the RK2 genome in the maintenance of plasmid RK2 in the broad spectrum of gram-negative bacteria. The results with the various plasmid constructs further show that while the *trfA* and *oriV* functions encode broad-host-range replication, there is no universal set of genetic determinants in plasmid RK2 that accounts for stable maintenance in all gram-negative bacteria. Instead, the observations lead us to conclude that gram-negative bacterial species differ in their requirements of various regions of plasmid RK2 for stable maintenance of this plasmid. In addition, the stability of IncP-1 plasmid maintenance may be related to the levels of expression of the *kil-kor* functions in each host. It is essential that the products of the maintenance regions be identified before we can understand the molecular basis of broad-host-range maintenance and the reasons for the different requirements shown by different bacterial species.

ACKNOWLEDGMENTS

We thank David Figurski, Don Guiney, and Gary Ditta for providing plasmids and helpful comments. We also thank Marcin Filutowicz, Alan Greener, Ross Durland, and Mike McEachern for critical reading of the manuscript.

This work was supported by Public Health Service grant A1-07194 from the National Institute of Allergy and Infectious Diseases and a grant from the National Science Foundation (no. PCM77-66573).

LITERATURE CITED

1. **Barth, P. T.** 1979. RP4 and R300B as wide host-range plasmid cloning vehicles, p. 399–410. *In* K. N. Timmis and A. Pühler (ed.), Plasmids of medical, environmental, and commercial importance. Elsevier/North-Holland Biomedical Press, Amsterdam.
2. **Barth, P. T., K. Ellis, D. H. Bechhofer, and D. H. Figurski.** 1984. Involvement of *kil* and *kor* genes in the phenotype of a host-range mutant of RP4. Mol. Gen. Genet. **197**:236–243.
3. **Bechhofer, D. H., and D. H. Figurski.** 1983. Map location and nucleotide sequence of *korA*, a key regulatory gene of promiscuous plasmid RK2. Nucleic Acids Res. **11**:7453–7469.
4. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
5. **Burkardt, H. J., G. Riess, and A. J. Pühler.** 1979. Relationship of group P1 plasmids revealed by heteroduplex experiments: RP1, RP4, R68, and RK2 are identical. J. Gen. Microbiol. **114**:341–348.
6. **Chikami, G. K., D. G. Guiney, T. J. Schmidhauser, and D. R. Helinski.** 1985. Comparison of 10 IncP plasmids: homology in the regions involved in plasmid replication. J. Bacteriol. **162**:656–660.
7. **Corbin, D., G. Ditta, and D. R. Helinski.** 1982. Clustering of nitrogen fixation (*nif*) genes in *Rhizobium meliloti*. J. Bacteriol. **149**:221–228.
8. **Ditta, G., T. Schmidhauser, E. Yakobson, P. Lu, X.-W. Liang, D. R. Finlay, D. Guiney, and D. R. Helinski.** 1985. Plasmids related to the broad host range vector pRK290 useful for gene cloning and for monitoring gene expression. Plasmid **13**: 149–153.
9. **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.

10. **Figurski, D. H., and D. R. Helinski.** 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided *in trans*. Proc. Natl. Acad. Sci. USA **76**:1648–1652.
11. **Figurski, D. H., R. Meyer, D. S. Miller, and D. R. Helinski.** 1976. Generation *in vitro* of deletions in the broad host range plasmid RK2 using phage Mu insertions and a restriction endonuclease. Gene **1**:107–119.
12. **Figurski, D. H., R. F. Pohlman, D. H. Bechhofer, A. S. Prince, and C. A. Kelton.** 1982. The broad host range plasmid RK2 encodes multiple *kil* genes potentially lethal to *Escherichia coli* host cells. Proc. Natl. Acad. Sci. USA **79**:1935–1939.
13. **Figurski, D. H., C. Young, H. C. Schreiner, R. F. Pohlman, D. H. Bechhofer, A. S. Prince, and T. F. D'Amico.** 1985. Genetic interactions of broad host range plasmid RK2: evidence for a complex replication regulon, p. 227–242. *In* D. R. Helinski, S. N. Cohen, D. B. Clewell, D. A. Jackson, and A. Hollaender (ed.), Plasmids in bacteria. Plenum Publishing Corp., New York.
14. **Fornari, C. S., and S. Kaplan.** 1982. Genetic transformation of *Rhodopseudomonas sphaeroides* by plasmid DNA. J. Bacteriol. **152**:89–97.
15. **Guiney, D. G.** 1982. Host range of conjugation and replication functions of the *Escherichia coli* sex plasmid Flac comparison with the broad host-range plasmid RK2. J. Mol. Biol. **162**:699–703.
16. **Guiney, D. G., and E. Yakobson.** 1983. Location and nucleotide sequence of the transfer origin of the broad host range plasmid RK2. Proc. Natl. Acad. Sci. USA **80**:3595–3598.
17. **Karoui, H., F. Bex, P. Dreze, and M. Couturier.** 1983. *Ham*22, a mini-F mutation which is lethal to host cell and promotes *rec*A-dependent induction of lambdoid prophage. EMBO J. **2**:1863–1868.
18. **Kornacki, J. A., A. H. West, and W. Firshein.** 1984. Proteins encoded by the trans-acting replication and maintenance regions of broad host range plasmid RK2. Plasmid **11**:48–57.
19. **Lanka, E., and P. T. Barth.** 1981. Plasmid RP4 specifies a deoxyribonucleic acid primase involved in its conjugal transfer and maintenance. J. Bacteriol. **148**:769–781.
20. **Meyer, R., D. Figurski, and D. R. Helinski.** 1977. Physical and genetic studies with restriction endonucleases on the broad host range plasmid RK2. Mol. Gen. Genet. **152**:129–135.
21. **Meyer, R., and D. R. Helinski.** 1977. Unidirectional replication of the P-group plasmid RK2. Biochim. Biophys. Acta **478**: 109–113.
22. **Meyer, R., and M. Hinds.** 1982. Multiple mechanisms for expression of incompatibility by broad-host-range plasmid RK2. J. Bacteriol. **152**:1078–1090.
23. **Ogura, T., and S. Hiraga.** 1983. Mini-F plasmid genes that couple host cell division to plasmid proliferation. Proc. Natl. Acad. Sci. USA **80**:4784–4788.
24. **O'Neill, E. A., C. Berlinberg, and R. A. Bender.** 1983. Activity of plasmid replicons in *Caulobacter crescentus*: RP4 and ColE1. Genetics **103**:593–604.
25. **Pohlman, R. F., and D. H. Figurski.** 1983. Essential genes of plasmid RK2 in *Escherichia coli*: *trfB* region controls a *kil* gene near *trfA*. J. Bacteriol. **156**:584–591.
26. **Schmidhauser, T. J., M. Filutowicz, and D. R. Helinski.** 1983. Replication of derivatives of the broad host range plasmid RK2 in two distantly related bacteria. Plasmid **9**:325–330.
27. **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.
28. **Selvaraj, G., and V. N. Iyer.** 1983. Suicide plasmid vehicles for insertion mutagenesis in *Rhizobium meliloti* and related bacteria. J. Bacteriol. **156**:1292–1300.
29. **Shapiro, J. A.** 1977. Appendix B: bacterial plasmids, p. 601–606. *In* A. I. Bukhari, J. A. Shapiro, and S. L. Adhya (ed.), DNA insertion elements, plasmids, and episomes. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
30. **Shingler, V., and C. M. Thomas.** 1984. Analysis of the *trf*A region of broad host range plasmid RK2 by transposon mutagenesis and identification of polypeptide products. J. Mol. Biol. **175**:229–249.
31. **Shingler, V., and C. M. Thomas.** 1984. Transcription in the *trf*A region of broad host range plasmid RK2 is regulated by *trf*B and *kor*B. Mol. Gen. Genet. **195**:523–529.
32. **Smith, C. A., V. Shingler, and C. M. Thomas.** 1984. The *trf*A and *trf*B promoter regions of broad host range plasmid RK2 share common potential regulatory sequences. Nucleic Acids Res. **12**:3619–3630.
33. **Smith, C. A., and C. M. Thomas.** 1983. Deletion mapping of *kil* and *kor* functions in the *trf*A and *trf*B regions of broad host range plasmid RK2. Mol. Gen. Genet. **190**:245–254.
34. **Smith, C. A., and C. M. Thomas.** 1984. Nucleotide sequence of the *trf*A gene of broad host range plasmid RK2. J. Mol. Biol. **175**:251–262.
35. **Smith, C. A., and C. M. Thomas.** 1984. Molecular genetic analysis of the *trf*B and *kor*B region of broad host range plasmid RK2. J. Gen. Microbiol. **130**:1651–1663.
36. **Stokes, H. W., R. J. Moore, and V. Krishnapillai.** 1981. Complemention analysis in *Pseudomonas aeruginosa* of the transfer genes of the wide host range R plasmid R18. Plasmid **5**:202–212.
37. **Thomas, C. M.** 1981. Complementation analysis of replication and maintenance functions of broad host range plasmids RK2 and RP1. Plasmid **5**:277–291.
38. **Thomas, C. M., and A. A. K. Hussain.** 1984. The *kor*B gene of broad host range plasmid RK2 is a major copy number control element which may act together with *trf*B by limiting *trf*A expression. EMBO J. **3**:1513–1519.
39. **Thomas, C. M., A. A. K. Hussain, and C. A. Smith.** 1982. Maintenance of broad host range plasmid RK2 replicons in *Pseudomonas aeruginosa*. Nature (London) **298**:674–676.
40. **Thomas, C. M., R. Meyer, and D. R. Helinski.** 1980. Regions of broad-host-range plasmid RK2 which are essential for replication and maintenance. J. Bacteriol. **141**:213–222.
41. **Thomas, C. M., C. A. Smith, V. Shingler, M. A. Cross, A. A. K. Hussain, and M. Pinkey.** 1985. Regulation of replication and maintenance functions of broad host range plasmid RK2, p. 261–276. *In* D. R. Helinski, S. N. Cohen, D. B. Clewell, D. A. Jackson, and A. Hollaender (ed.), Plasmids in bacteria. Plenum Publishing Corp., New York.
42. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal promoters. Gene **19**:259–268.
43. **Watson, B., T. C. Currier, M. P. Gordon, M.-D. Chilton, and E. W. Nester.** 1975. Plasmid required for virulence of *Agrobacterium tumefaciens*. J. Bacteriol. **123**:255–264.
44. **Young, C., D. H. Bechhofer, and D. H. Figurski.** 1984. Gene regulation in plasmid RK2: positive control by *korA* in the expression of *korC*. J. Bacteriol. **157**:247–252.