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Essential Genes of Plasmid RK2 in *Escherichia coli*: *trfB* Region Controls a *kil* Gene Near *trfA*

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Plasmid RK2 encodes several *kil* determinants whose lethal action on *Escherichia coli* host cells is prevented by RK2 *kor* genes. Here we show that the mini-RK2 plasmid, pRK248, specifies a *kilB* component (*kilB1*) in the region of the replication gene *trfA*. *kilB1* is different from *trfA* and is completely encoded within the pRK248 *Hae*II A fragment. Transformation of *E. coli* cells with hybrid plasmids containing the cloned *kilB1* determinant is very inefficient and results in the selection of variant kil^- plasmids, many of which show genetic and physical evidence of deletions. If another pRK248 gene (*korB1*) is present in the cells, $kilB1^+$ plasmids can be established at high efficiency and without any detectable changes. *KorB1* is encoded by the *trfB* region of pRK248 because recombinant plasmids with this region are able to control *kilB1* in *trans*. These results substantiate our earlier explanation for the structure of pRK248 and for the perplexing requirement of the *trfB* region in this plasmid.

Two classes of genetic determinants are essential to plasmid RK2 (12) maintenance in *Escherichia coli*. One class, as expected, specifies the information required for plasmid replication and its control. Two such determinants have been clearly identified for RK2: *oriV* and *trfA*. *OriV* is the origin of replication, originally determined by electron microscopic analysis of replicating intermediates (14). Genetically, *oriV* behaves as a *cis*-acting replication determinant that is activated by a diffusible function (6) specified by *trfA* (22). Thus, the *trfA* product is a positive-acting element in RK2 replication, but its function is not known.

The second class of essential RK2 genes is comprised of *korA*, *korB*, and *korC* (10). These are required to prevent the lethal action of the RK2 *kilA*, *kilB*, and *kilC* genes on the *E. coli* host. The functions of these peculiar genes are as yet unknown, but the *kil* determinants are nonessential to RK2 in *E. coli* (10, 17a). Thus, the corresponding *kor* genes can be considered only conditionally essential in this host, i.e., *kor* is required only when *kil* is present. In this way they are distinct from *E. coli*-specific replication determinants. However, the *kor* genes (and probably *kil*) are highly conserved among plasmids in the same incompatibility group (IncP) as RK2, and we have suggested that they are involved in the broad host range typical of these plasmids (5, 17).

This work concerns a region of RK2 thought to specify a third replication determinant designated *trfB*. In plasmid pRK248, a small derivative of RK2, the *trfB* region appears to be essential (22). Mutants with defects in the *trfB* region are maintained poorly, if at all, in *E. coli*, and these can be complemented by providing the cloned *trfB* region in *trans*. However, other studies have shown that with certain plasmids the *trfB* region is dispensable for RK2 replication (17a, 20).

We have proposed an explanation for these seemingly incongruous results (9, 10, 17a). We predicted (i) that pRK248 encodes a nonessential *kil* gene close to *trfA* and (ii) that the plasmid also specifies the required *kor* determinant in the *trfB* region. Thus, the requirement for *trfB* region in RK2 derivatives would actually reflect whether *kil* is present, rather than a need for a third replication function. In the studies reported here, we show these predictions to be true. A preliminary report of this work has been presented (9).

MATERIALS AND METHODS

Nomenclature. If a relevant plasmid gene is not present in a bacterial strain, we indicate this with a superscript 0 (e.g., $korB1^0$). Coordinates of the RK2 physical map are defined by the distance from the *Eco*RI site in kilobases and are designated by a prime (′) (e.g., 14′ to 22′ region).

Bacterial strains and plasmids. *E. coli* MV10 is C600

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Δ*trpE5* (6) from C. Yanofsky; C2110 is *E. coli* C *his rha polA1* provided by M. Kahn. The plasmids are described in Table 1.

Media, enzymes, and standard procedures. Media for growth and selection of bacteria have been described previously (8, 10). Restriction enzymes and T4 DNA ligase were purchased from commercial suppliers and used as suggested. Preparation of plasmid DNA (13), agarose gel electrophoresis (13), and transformation of *E. coli* with plasmid DNA (3) were done by the published procedures.

Extraction of DNA from low-melting-temperature agarose gel. After electrophoresis through 0.6% low-melting-temperature agarose gels, slices with the DNA bands were mixed with 1.0 ml of 100 mM Tris-hydrochloride–1 mM disodium EDTA buffer (pH 7.4) and heated to 65°C for 30 min. The mixture was extracted three times with phenol (saturated with 1.0 M Tris-hydrochloride, pH 7.4) and two times with chloroform-isoamyl alcohol (24:1). The aqueous phase was brought to 0.3 M potassium acetate, and the DNA was precipitated at −20°C with 2 volumes of ethanol.

Radiolabeling of DNA and DNA-DNA hybridization. Gel-purified DNA fragments were labeled with α^{32}P-nucleotides by nick translation with DNA polymerase I by the method of Rigby et al. (18). Specific activity ranged between 10^7 and 10^8 dpm/μg of DNA. Agarose gel-separated DNA was blotted to nitrocellulose and hybridized to ^{32}P-labeled DNA by published procedures (19). The colony hybridization protocol was that of Grunstein and Wallis (11). For autoradiography we used Kodak XAR-5 X-ray film with Kodak-Cronex intensifying screens.

RESULTS

Genetic evidence that a *kil* determinant is in the *trfA* region of pRK248. The physical relationship of the mini-RK2 plasmid pRK248 to parental RK2 is shown in Fig. 1. To explain the essential nature of the *trfB* region for pRK248 maintenance in *E. coli* (20, 22), we postulated that the *trfA* region carries a lethal *kil* gene and that the *trfB* region encodes the *kor* function required for its control (9, 10, 17a). Since the *trfA* region of pRK248 overlaps the *kilB* region of RK2, we expected that any *kil* gene present on pRK248 would be related to the *kilB* determinant; similarly, the *kor* gene was expected to involve *korB*.

Our attempts to detect *korB* on pRK248 were negative; pRK248 could not support the maintenance of the *kilB*$^+$ test plasmids (D. Bechhofer and D. Figurski, manuscript in preparation). However, we (incorrectly, but fortunately) reasoned that perhaps the expression of *korB* from pRK248 was too low to support the high-copy-number *kilB*$^+$ plasmids, and so we continued with a second approach. This was to inactivate the putative *korB* in pRK248 to reveal *kil*. Earlier (10), we showed that *korB* could be eliminated

TABLE 1. Plasmids

Plasmid	Relevant phenotype	Relevant genotype	Description	Reference
pCT16	Km^r	*trfB*$^+$ *korB1*$^+$	Mini-ColE1 replicon with *trfB* region of pRK248	Thomas et al. (22)
pDB1	Gm^r Trp$^+$	*trpE*$^+$	Mini-ColE1-*trpE* plasmid which gives a Gm^r-encoding *Sst*II fragment	Bechhofer and Figurski, unpublished data
pKJ1	Ap^r		P15A replicon with a single *Hae*II site derived from pACYC177	Figurski, unpublished data
pLB2	Tp^r IncW		Mini-plasmid with replication region of R388	Babiss and Figurski, unpublished data
pRK248	Tc^r	*oriV*$^+$ *trfA*$^+$ *trfB*$^+$ *kilB1*$^+$ *korB1*$^+$	Mini-RK2 replicon (Fig. 1)	Thomas et al. (22)
pRK2104	Ap^r	*korA*$^+$ *korB*$^+$ *korB1*$^+$	Mini-ColE1 replicon with the 0′ to 8.5′ and 50.4′ to 56.4′ region of RK2	Figurski et al. (10)
pRK2108	Ap^r Trp^{+r}	*korA*$^+$ *korB*$^+$ *korB1*$^+$ *trpE*$^+$	pSM1 replicon with the 50.4′ to 56.4′ region of RK2	Figurski et al. (10)
pRK2149	Ap^r	*oriV*$^+$	P15A replicon with the *oriV* of RK2 and a single *Hae*II site	Spivak and Figurski, unpublished data
pRK2164	Tc^r Gm^r Kil$^+$	*trfA*$^+$ *kilB1*$^+$	Hybrid of pDB1 and the Tc^r-*trfA* region of pRK248 (Fig. 1)	This work
pRK2192	Ap^r Kil$^+$	*oriV*$^+$ *kilB1*$^+$	pRK2149 with *Hae*II-A of pRK248	This work
pRK2194	Ap^r	*oriV*$^+$	pRK2149 with *Hae*II-D of pRK248	This work
pRK2195	Ap^r	*oriV*$^+$	Same as pRK2194, but with *Hae*II-D in opposite orientation	This work
pRK2198	Km^r	*trfB*$^+$ *korB1*$^+$	Hybrid of pSM1 and pCT16 at their *Eco*RI sties	This work
pRK2199	Ap^r	*oriV*$^+$ *trfA*$^+$ *kilB1*$^+$	pRK2149 with *Hae*II-A and -D fragments of pRK248	This work
pSM1	IncFII		Spontaneous minivariant of R100	Mickel and Bauer (16)

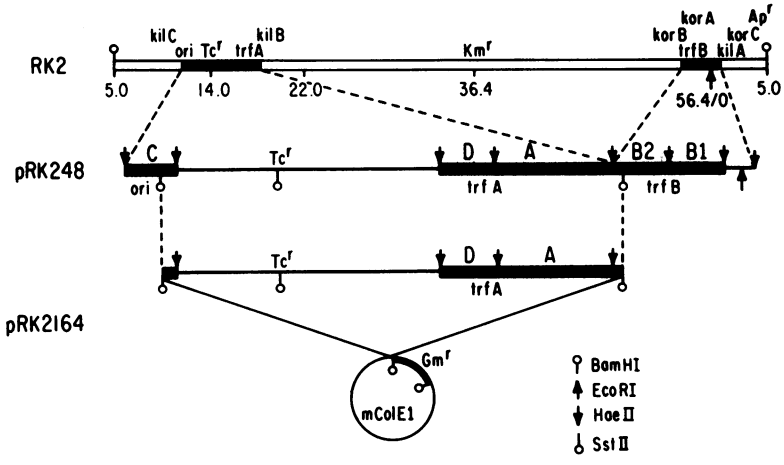

FIG. 1. Relationship of pRK248 to RK2 and pRK2164. The genetic and physical map of RK2 (56.4 kilobases) is linearized at its *Bam*HI site at coordinate 5.0′. The numbers refer to RK2 coordinates in kilobase units from the *Eco*RI site. Only relevant restriction endonuclease cleavage sites are shown. The regions of RK2 present in pRK248 (22) are expanded and flanked by dashed lines. Of the multiple *Hae*II fragments in pRK248 (22), only those delineating the *oriV* segment (C), the *trfA* region (A and D), and the *trfB* region (B1 and B2) are shown. pRK2164 consists of two contiguous *Sst*II fragments of pRK248 inserted at one of the *Sst*II sites of pDB1 (circle).

by interruption of the *Sst*II site at 54′ on RK2. Because the plasmid pRK248 has this site in its *trfB* region, we attempted to interrupt it with a Gm^r-encoding *Sst*II fragment. Such a plasmid should be constructed in a *korB*$^+$ host and tested in a *korB*^{0} host.

Plasmid pRK248 was digested partially with *Sst*II and ligated to *Sst*II-cleaved pDB1, a ColE1 plasmid which yields a Gm^r-encoding *Sst*II fragment. This mixture was used to transform a host containing pRK2104, a ColE1 plasmid which is *korB*$^+$ and confers Ap^r. Selection was for Tc^r Gm^r Ap^r colonies. Only three transformants were isolated, and none had plasmids with the expected structure. It was surprising that all three plasmids were hybrids of pDB1 and fragments from pRK248, because it was expected that this combination would be incompatible with the ColE1 plasmid pRK2104. One of these, pRK2164, was useful (Fig. 1). This plasmid contains both *Sst*II fragments required for expression of Tc^r and therefore includes a nonfunctional remnant of the *oriV* region and a 150-base-pair segment from the *trfB* region.

Because pRK2164 carries the *trfA* region of the mini-RK2 plasmid pRK248 in the absence of a substantial portion of the *trfB* region, we tested it for the expression of any Kil-like phenotype. A Kil$^+$ phenotype should be evident as the inability of plasmid pRK2164 to transform cells which lack the required *kor* determinant of RK2. In this experiment, we expected that any *kil* determinant on pRK2164 would be related to *kilB* and that any required *kor* would be *korB*. Thus, we compared the ability of pRK2164 to transform *korB*^{0} cells (i.e., lacking *korB*) and *korB*$^+$ cells. The *korB*$^+$ determinant was provided by plasmid pRK2108, a pSM1 derivative with the *korB* region of RK2 and the selective marker *trpE*. The plasmid DNA used in these transformations was extracted from the original clone which gave rise to pRK2164, and thus the preparation also included the ColE1 *korB*$^+$ Ap^r plasmid pRK2104.

Transformation of the *korB*$^+$ strain to Tc^r, the marker on pRK2164, was efficient; all colonies appeared normal. Transformation of the *korB*^{0} strain was less efficient and produced two colony types: normal-appearing large colonies and small colonies with irregular edges.

All large colonies were Ap^r. These were double transformants carrying not only pRK2164, but also pRK2104, the *korB*$^+$ plasmid in the DNA preparation. Small colonies were Ap^s and thus did not contain the *korB*$^+$ plasmid. Only a few of the small colonies were viable after restreaking. Those that were gave rise to large, healthy-appearing variants. In addition to the difference in colony morphology, these surviving cells showed evidence of other genetic changes (Table 2). About 25% no longer expressed the Gm^r determinant of pRK2164. Many of the survivors showed differences in the *trfA* activity which should be expressed by the pRK2164 plasmid. (The *trfA* function is easily detected as the ability to support the replication of an *oriV*-containing plasmid, such as pRK2128, which carries only *oriV* and a Cm^r determinant.) In contrast, neither Gm^r nor *trfA* was lost from the pRK2164 transformants of

TABLE 2. Genetic and physical properties of *korB*-independent variants of pRK2164[a]

Strain	Genetic markers		Physical markers	
	Gm[b]	TrfA[c]	*Hae*II-A[d]	*Hae*II-D[e]
RP1279	R	+	Δ	+
RP1323	R	+	+	+
RP1324	R	+	Δ	+
RP1325	R	+	+	+
RP1326	R	−	Ω	+
RP1327	S	−	−	−
RP1328	R	+	+	+
RP1329	R	+	+	+
RP1330	R	+	+	+
RP1331	S	−	−	−
RP1332	S	−	−	−
RP1333	R	+	Δ	+
RP1334	S	−	−	−
RP1335	R	+	+	+
RP1336	R	−	−	−

[a] MV10 (*korB*^{0}) strain was transformed with DNA taken from RP1229, MV10(pRK2164, pRK2104). Tc^r Pn^s transformants were isolated and screened for Gm^r and the ability to *trans* complement an RK2 *oriV*$^+$ plasmid. Plasmid DNA taken from these strains was digested with *Hae*II and analyzed by ethidium bromide-gel electrophoresis and by filter hybridization analysis with *Hae*II A and *Hae*II D fragment-specific probes (Fig. 2).

[b] R and S, Resistance and sensitivity to gentamicin, respectively.

[c] + and −, Ability and inability to *trans* complement RK2 *oriV*$^+$ plasmids, respectively.

[d] +, Presence of a normally migrating *Hae*II A fragment; −, absence of the *Hae*II A fragment; Δ, change in the migration properties of the *Hae*II A fragment; Ω, interrupted *Hae*II A fragment.

[e] + and −, Presence and absence of the *Hae*II D fragment, respectively.

korB$^+$ cells. The pRK2164 plasmid derivatives isolated from the variant colonies are able to transform both *korB*^{0} and *korB*$^+$ cells with equal efficiencies and with no differences in colony morphology. Thus, these plasmids are genetically different from the parental pRK2164.

One possible explanation for these results is that pRK2164 carries a *kil* determinant which is under the control of a *kor* gene in the *korB* region. The *kil* determinant could be located between *trfA* and the Gm^r determinant on pRK2164 (Fig. 1). In the absence of *kor*, the putative *kil* function stresses the *E. coli* host and generates selective pressure for loss of the *kil*. Some of the *kil*$^-$ mutations might be deletions which include *trfA* or the Gm^r determinant or both. We show below that this hypothesis is correct.

Structural changes of pRK2164 derivatives isolated from *kor*^{0} cells. The putative *kil* determinant is expected to be in *Hae*II fragments A or D of the mini-RK2 plasmid pRK248 (Fig. 1) because these define the *trfA* region (22). Therefore the plasmids were analyzed with respect to their *Hae*II cleavage patterns. Although analysis of ethidium bromide-stained gels is difficult because of the many *Hae*II fragments released from these plasmids, we were able to make some general observations.

We predicted above that at least those pRK2164 plasmid derivatives which had lost another marker (*trfA* or Gm^r) should show evidence of deletion. Consistent with this is our finding that the *Hae*II A fragment was always missing from plasmids of this class. In addition, some *trfA*$^+$ Gm^r plasmids had also lost the fragment. The *Hae*II D fragment was not detectable in some of the derivatives, and these were invariably *trfA*$^-$. All *trfA*$^+$ plasmids showed *Hae*II-D to be intact.

This pattern was confirmed by Southern blot hybridization analysis with ^{32}P-labeled *Hae*II A and D fragments (Fig. 2 and Table 2). As expected, pRK2164 from a *korB*$^+$ host has normally migrating *Hae*II A and D fragments. The variants of pRK2164 isolated from a *kor*^{0} host did show differences. A normal *Hae*II D fragment was detected in plasmids which were *trfA*$^+$; hybridization was evident with only one of the

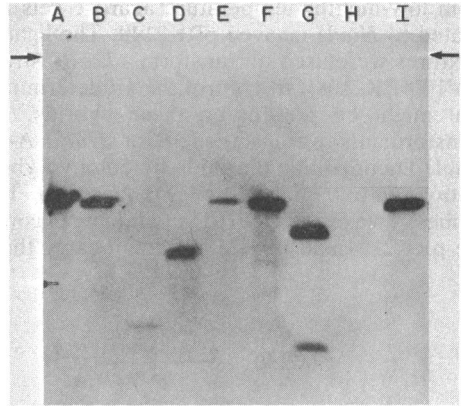

FIG. 2. *Hae*II fragment variation in pRK2164 plasmids from *korB*^{0} cells. Plasmid DNA was purified from the strains listed in Table 2. After digestion with *Hae*II, the DNA was separated by electrophoresis in 1.4% agarose gels and then transferred to nitrocellulose for hybridization to ^{32}P-labeled *Hae*II A fragment. Bands of hybridization were visualized by autoradiography, and a summary of the results is presented in Table 2. Shown here are the patterns for selected plasmids characteristic of the various classes we found. The pRK2164 control plasmid, isolated from *korB*$^+$ pRK2104-containing strain, is shown in lane A. pRK248 is in lanes F and I. The other plasmids are from the following strains: B, RP1323 (Gm^r TrfA$^+$); C, RP1333 (Gm^r TrfA$^+$); D, RP1279 (Gm^r TrfA$^+$); E, RP1329 (Gm^r TrfA$^+$); G, RP1326 (Gm^r TrfA$^-$); and H, RP1327 (Gm^s TrfA$^-$). Arrows indicate sample origin.

trfA$^-$ plasmids (see below). *Hae*II fragments which hybridized to the *Hae*II-A probe were not always the size of *Hae*II-A. The new A-specific fragments are the expected remnants of *Hae*II A fragment now fused to DNA at the other endpoint of the deletion. All but one of the plasmids that had lost *trfA* failed to hybridize both *Hae*II-A and *Hae*II-D probes. The one variant is the plasmid from the transformant designated RP1326. This plasmid is *trfA*$^-$, but showed an intact *Hae*II-D and two new bands that hybridized to the *Hae*II-A probe. We interpret this to be the result of an insertion in the *Hae*II A fragment.

The genetic and physical characterization of these *kor*-independent variants of pRK2164 shows that the putative *kil* determinant is very likely within the *Hae*II A fragment and adjacent to *trfA*.

***kil* is expressed by the cloned *Hae*II A fragment.** To test our predictions on the location of *kil*, we cloned the *Hae*II A and D fragments of pRK248. The vehicle used was pRK2149 (Fig. 3), which is an Ap^r-encoding P15A replicon with a single *Hae*II site and the *oriV* segment of RK2. The *oriV* segment was important to the cloning of both *Hae*II A and D fragments onto the same plasmid.

*Hae*II A and D fragments were each purified from low-melting-temperature agarose gels and ligated to *Hae*II-cleaved pRK2149. The ligation mixtures were used to transform a *korB*$^+$ strain, MV10(pRK2108), to control the *kil* determinant that might be present on these hybrids. Ap^r transformants were screened for *Hae*II-A- or *Hae*II-D-containing plasmids by colony hybridization with ^{32}P-labeled *Hae*II-A or *Hae*II-D probes. Two of the *Hae*II-D-containing plasmids are pRK2194 and pRK2195, which carry the D fragment in opposite orientations; pRK2192 is the only hybrid plasmid we isolated with *Hae*II A fragment alone. The structures of these plasmids are shown in Fig. 3.

To test for the presence and expression of a *kil* determinant on these hybrid plasmids, we looked for differences in the efficiency of transformation of *kor*^{0} and *kor*$^+$ hosts. Plasmids containing *kil* determinants cannot be maintained in *kor*^{0} hosts because the uncontrolled *kil* determinant is lethal to the host. The results in Table 3 clearly demonstrate that colonies are not formed after transformation of the *kor*^{0} host with plasmid pRK2192. However, the *kor*$^+$ hosts are transformed efficiently by this plasmid. Furthermore, no other plasmid showed this failure to transform the *kor*^{0} host. Because pRK2192 has the *Hae*II A fragment of pRK248, we conclude that this fragment encodes a *kil* determinant. The *Hae*II-A fragment originates from the *kilB* region of RK2 and very likely contributes to the Kil phenotype expressed by that region (10). Therefore, we have named this determinant "*kilB1*."

The results in Table 3 demonstrate that pRK2198 can provide the required *kor* function that allows the *kilB1*$^+$ pRK2192 plasmid to be maintained. The only portion of RK2 present in pRK2198 is the *trfB* region. Thus, we conclude that *trfB* region encodes the *kor* determinant for *kilB1*. In keeping with our nomenclature for *kil* and *kor* genes (10), this determinant is called "*korB1*."

***kilB1* is distinct from *trfA*.** These plasmids were also tested for *trfA* by transformation of *polA*$^-$ hosts, in which the P15A replicon of the pRK2149 vehicle is inactive (2). Because RK2 replication is *polA*$^+$ independent (7), the hybrid plasmid will be maintained if it carries the repli-

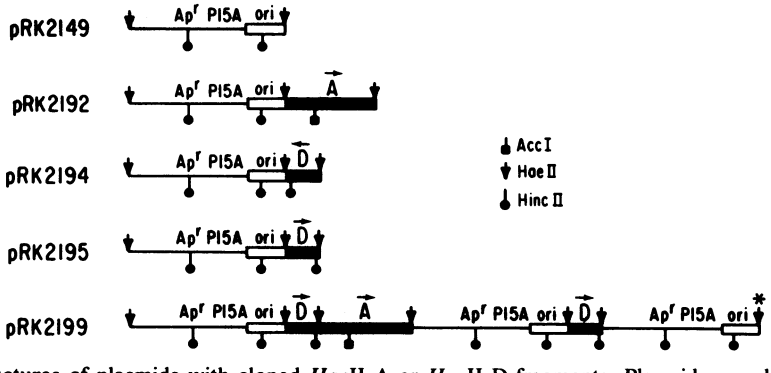

FIG. 3. Structures of plasmids with cloned *Hae*II A or *Hae*II D fragments. Plasmids are aligned with the pRK2149 vehicle portion linearized at its *Hae*II site. Arrows show the relative orientations of the inserted fragments. "Ori" designates the *OriV* region of RK2. "P15A" shows the location of the P15A replication region. In pRK2199, the second *Hae*II D fragment could also be in the site marked with an asterisk, since our results do not distinguish between these two alternatives. The construction of these plasmids is described in the text.

TABLE 3. Expression of the Kil phenotype by the cloned *Hae*II A fragment

Transforming plasmid	Relative efficiency of transformation[a] in recipient strains		
	No helper (*korB*^{0})	pRK2108 helper (*korB*$^+$)	pRK2198 helper (*korB1*$^+$)
pRK2149	1.3	1.0	1.5
pRK2192	<0.0002	1.0	2.0
pRK2194	1.5	1.0	2.0
pRK2195	1.3	1.0	2.0
pRK2199	(1.2)[b]	1.0	0.4

[a] MV10, MV10(pRK2108), and MV10(pRK2198) strains were transformed with the various plasmids, and penicillin-resistant colonies were selected. Efficiencies of transformation for each plasmid are normalized to the MV10(pRK2108) strain. The relative competence of the strains was monitored as described previously (10) by transformation with pKJ1. Values are adjusted for competence differences (never more than twofold).

[b] Because of the internal homology in pRK2199, *Hae*II A fragment-deficient plasmids can be easily formed by recombination. In contrast to the results for the other two strains, transformants of the *korB*^{0} MV10 strain never yielded intact pRK2199 plasmid.

cation gene *trfA*, whose product will activate the RK2 *oriV* sequence on the pRK2149 segment. Table 4 shows that the *Hae*II-A plasmid pRK2192 and the *Hae*II-D plasmids pRK2194 and pRK2195 cannot transform the *polA*$^-$ host, even when *korB1* is provided to control any *kilB1* activity which might be expressed.

This is consistent with results of the preceding sections and those of Thomas et al. (22), which indicate that both *Hae*II A and D fragments are required for functional *trfA*. We substantiated this further by constructing a *trfA*$^+$ hybrid plasmid from a ligation of *Hae*II-cleaved pRK2192 and pRK2195. This plasmid, pRK2199, was isolated by direct selection for *trfA*$^+$ in a *polA*$^-$ host (which carried the *korB1*$^+$ plasmid pRK2198). The salient feature of the structure of pRK2199 (Fig. 3) is the presence of *Hae*II A and D fragments in their parental configuration.

Therefore, the *Hae*II A and D fragments are both required for *trfA*, whereas *Hae*II-A is sufficient to express *kilB1*.

DISCUSSION

We can now provide a plausible explanation for the structure of plasmid pRK248 and for the quasi-essential nature of the *trfB* region. This is based on our finding that the *trfA* region of pRK248 encodes a *kil*-like determinant, *kilB1*, whose expression can be detrimental to the *E. coli* host cell. The necessary regulation of *kilB1* is mediated by another plasmid gene, *korB1*, and this maps in the *trfB* region.

Our cloning studies prove that the replication determinant *trfA* requires both the *Hae*II A and D fragments of pRK248. This is consistent with the analysis of Thomas et al. (22), who showed that deletion of *Hae*II-D inactivated the pRK248 replicon and that no replication-proficient derivatives lacking *Hae*II-A or -D could be isolated. In addition, we find that *kilB1* is specified by the *Hae*II A fragment alone. This close linkage of *kilB1* to *trfA* is important to understanding the structure of pRK248, which was constructed in vitro from a much larger RK2 derivative.

Plasmid pRK248 was most likely formed by the ligation of two separate fragments resulting from a partial *Hae*II digestion of the considerably larger plasmid, pRK229 (22). One fragment contains *oriV*, *trfA*, and the Tc^r determinant and is presumably the 11′ to 19′ region of RK2. The other fragment carries the 54′ to 56.4′, 0′ to 0.3′ region, of which the 54′ to 56.4′ portion is defined as *trfB* (22). Because a replication-proficient plasmid was selected in the transformation, *oriV* and *trfA* are present. The need for *trfA* necessitates the inclusion of both *Hae*II fragments A and D, so the selection also guaranteed that *kilB1* would be carried by the plasmid. This in turn adds the requirement for *korB1* to prevent uncontrolled *kilB1* action on the *E. coli* host. Since we find that *korB1* is encoded by the *trfB* region, we conclude that one reason that the *trfB* region is essential to pRK248 is to provide *korB1*.

The *trfB-korB1* region is clearly important for normal maintenance for *kilB1*$^+$ plasmids replicating by the RK2 replication system. The pRK2199 plasmid constructed here is a P15A plasmid which also carries the RK2 determi-

TABLE 4. Expression of *trfA* function by cloned *Hae*II A and *Hae*II D fragments

Transforming plasmid	Relative efficiency of transformation[a] in recipient *polA*$^-$ strains	
	No helper (*korB1*^{0})	pRK2198 helper (*korB1*$^+$)
pRK2149	<0.002	<0.002
pRK2192	<0.002	<0.002
pRK2194	<0.002	<0.002
pRK2195	<0.002	<0.002
pRK2199	(2.4)[b]	1.0

[a] The *polA*$^-$ strains C2110 and C2110(pRK2198) were transformed with each of the plasmids. Relative competence was determined by transformation with equal amounts of pLB2 plasmid. The efficiencies of transformation are all normalized to that of C2110(pRK2198) by pRK2199.

[b] These transformants were tiny and of variable size.

nants *oriV*, *trfA*, and *kilB1*, but not *korB1* or *trfB*. In *polA*$^-$ cells, where the P15A replicon is inactive, pRK2199 will replicate, but the cells grow very poorly and give rise to small, variable-sized colonies. Supplying the *trfB-korB1* region in *trans* restores normal growth characteristics. A very similar phenotype was reported for pCT6 constructed by Thomas et al. (22). They interpreted their results to indicate that *trfB* region encodes a function directly involved in plasmid replication or maintenance.

We have tested this hypothesis. If *trfB* codes for an important replication function in addition to the *korB1* determinant, then *trfB* should be required even in the absence of *kilB1*. Our results show that this is not the case. By using strains carrying *trfA*$^+$ *kilB*(*ts*) plasmids at the nonpermissive temperature for *kilB* function, we have observed that an *oriV*-containing plasmid can be maintained in the absence of any *trfB*$^+$-*korB*$^+$ helper plasmid (17a). In the work presented here, we show further that *kilB1* can be lost from a *trfA*$^+$ *kilB1* plasmid by spontaneous mutation. These resulting *trfA*$^+$ *kilB1*$^+$ derivatives are also capable of supporting an *oriV*-containing plasmid in the absence of the *trfB-korB1* region. In addition, we have recently constructed an *oriV*$^+$ recombinant of one such *trfA*$^+$ *kilB1*$^-$ plasmid (H. Schreiner, R. Pohlman, D. Bechhofer, C. Young, P. Borden, and D. Figurski, manuscript in preparation). This plasmid is missing the ColE1 replicon and the *trfB-korB1* region. It replicates with a copy number approximately equal to that of pRK248, and the cells containing the plasmid grow normally. Thus, we conclude that the *trfB-korB1* region is not essential for RK2 replication in *E. coli*.

Thomas (20) has reported a *trfA*$^+$ *trfB*$^-$ plasmid that will complement an *oriV* plasmid in *trans*. The *trfA*-encoding segment, termed "*trfA**," carries transposon Tn*5* sequences, and it is not clear whether the Tn*5* remnant is responsible for the *trfB* independence. However, in view of the results presented here and the fact that the Tn*5* insertion occurred in the *Hae*II A fragment, it seems very likely that this plasmid is *kilB1*$^-$ and therefore has no need for *korB1*.

It is important to note that *kilB1*$^-$ variants of *kilB1*$^+$ plasmids can be selected easily. We showed that transformation of *kor*^{0} cells with the *kilB1*$^+$ plasmid pRK2164 produces small, struggling colonies. Papillae of rapidly growing cells will often emerge, and these cells are the source of the *kilB1*$^-$ plasmid variants. Presumably the full effect of *kilB1* on a *kor*^{0} host is not always immediate, and there is opportunity for cells to emerge with *kilB1*$^-$ plasmids. In addition, we find that some of the *kilB1*$^-$ mutations affect expression of *trfA*. For example, we have isolated spontaneous *kilB1*$^-$ derivatives of pRK2164 which express *trfA*, but poorly compared to pRK2164 itself (R. Pohlman and D. Figurski, unpublished data). Thus, we urge caution in the interpretation of results with *trfA*$^+$ plasmids constructed from *trfA*$^+$ *kilB1*$^+$ parents in the absence of *korB1*. Furthermore, it is conceivable that the effects of spontaneous mutants of *kilB1* may be undetectable in *E. coli*, but may become significant when the plasmids are introduced into other gram-negative bacteria.

The properties expressed by the *kilB1* and *korB1* determinants have revealed new information about *kilB* and *korB*. *kilB* is the host-lethal determinant encoded by the 14′ to 22′ region of RK2, and it is controlled by *korB* which maps in the 50.4′ to 56.4′ region (10). Since the *kilB1* determinant of pRK248 maps within the *kilB*-encoding region of RK2, we expected that *kilB1* is responsible for the lethal effect of the *kilB* region. However, *korB1* from pRK248 (or the analogous region from parental RK2) will control *kilB1* from pRK248, but it will not allow transformation by a plasmid carrying the complete 14′ to 22′ *kilB* region of RK2 (Bechhofer and Figurski, in preparation). This is not a copy effect because high-copy-number *korB1*$^+$ plasmids fail to control *kilB*, whereas a single copy of *korB* integrated in the host chromosome is sufficient. The *kilB1* determinant isolated from pRK248 is not an attenuated *kilB* determinant because we have cloned (in a *korB*$^+$ host) the analogous *Hae*II fragment from a *kilB*$^+$ plasmid (Pohlman and Figurski, unpublished data). Its phenotype with respect to *korB1* dependence, *korB* independence, and effect on host cell viability is indistinguishable from that of the *kilB1* from pRK248. Thus *korB1* is lacking the determinants for complete *korB* activity, and *kilB1* is insufficient to account for the phenotype specified by the *kilB* region.

Therefore, we feel that the *kilB1* determinant is only one component of *kilB* and that *korB1* is specific for that component. This suggests that at least one other *kilB* determinant is present in the 14′ to 22′ region and that this will require the determinant(s) of *korB* that is not present in *korB1*. Consistent with this prediction is our finding that *korB* function requires determinants which map outside the *korB1* (*trfB*) region studied here (Bechhofer and Figurski, in preparation).

What are the true functions of *kilB1* and *korB1*? All of the known *kil* and *kor* genes are nonessential for replication in *E. coli*. However, because these genes are present on all IncP plasmids tested and are unique to the IncP group, we postulated (10) their involvement in the broad host range which characterizes this

group of plasmids (17). At present there is no direct evidence to support this contention, but we are intrigued by several observations: (i) the proximity of *kilB1* to *trfA* (this work); (ii) the location of host-range mutations near *korA-korB* (1; Bechhofer and Figurski, unpublished data), *kilB1* (4), and *kilC* (4); and (iii) an apparent involvement of the *trfB-korA-korB1* region in the replication of certain, but not all, derivatives of pRK248 in *Pseudomonas* spp. (18a, 21). Furthermore, there is evidence that certain *kil* and *kor* genes may act as accessory functions for plasmid replication, even in *E. coli*. Meyer and Hinds (15) reported a new incompatibility determinant that maps in the *korA-korB1* region. They interpreted their results to indicate a direct role for *kilA* and *korA* in RK2 maintenance. In addition, we have found that *kilB1* and *korB* can have a significant effect on RK2-specific replication in *E. coli* (H. Schreiner et al., in preparation). Thus, the true reason for the existence of *kil* and *kor* genes is beginning to emerge, although for the most part these determinants remain a curious mystery. We are seeking a full understanding of their interactions and functions.

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