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# Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in *trans*

(plasmid replication/replication origin/*trans*-complementation/broad host range/gene cloning)

DAVID H. FIGURSKI* AND DONALD R. HELINSKI

Department of Biology, University of California at San Diego, La Jolla, California 92093

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**ABSTRACT** **pRK212.2, a derivative of the broad host range plasmid RK2, contains two *Eco*RI cleavage fragments, A and B, neither of which can replicate by itself in *Escherichia coli*. Fragment A (41.7 kilobases), but not fragment B (14.4 kilobases), can be cloned by insertion into the unrelated plasmids mini-F and ColE1. Fragment B contains the origin of replication and the ampicillin-resistance determinant of RK2. Transformation of *E. coli* cells containing the mini-F–fragment A hybrid plasmid with fragment B DNA results in the recircularization and replication of fragment B as a nonmobilizable plasmid (pRK2067) with the copy number and incompatibility properties of RK2. Fragment B cannot be cloned in the absence of fragment A because the latter fragment suppresses a function, specified by fragment B, that results in loss of host cell viability. A small segment (2.4 kilobases) of fragment B that contains the RK2 origin of replication but no longer affects host cell growth in the absence of fragment A had been cloned previously by insertion into a ColE1 plasmid. This hybrid plasmid, designated pRK256, will replicate in *E. coli polA* mutants only when a fragment A-bearing helper plasmid is present. These results demonstrate that the potentially lethal function specified by fragment B of RK2 is not necessary for replication and that at least one *trans*-acting function is directly involved in RK2 replication.**

Bacterial plasmids of the P-1 incompatibility group (Inc P-1) are capable of replication in a wide range of Gram-negative bacteria (1–4). We have been examining the replication properties of RK2, an Inc P-1 antibiotic-resistance plasmid (5), in an effort to determine the molecular basis of its broad host range. RK2 [56.4 kilobases (kb)] is self-transmissible and codes for resistance to kanamycin (Km), ampicillin (Ap), and tetracycline (Tc) (5, 6). In *Escherichia coli* the molecule replicates unidirectionally from a fixed origin located approximately 12 kb from the single *Eco*RI cleavage site (7). From studies of RK2 molecules manipulated *in vitro* with restriction endonucleases, it appears that the genes essential for replication and maintenance in *E. coli* are distributed over a 24-kb portion of the plasmid that includes the origin of replication (ref. 8; unpublished data). This finding is in contrast to the considerably smaller replication regions of plasmids of other incompatibility groups that appear to have a more limited host range than does RK2.

To locate and characterize individual genes required for replication, the RK2 essential region has been further subdivided by the construction of hybrid plasmids carrying RK2 genes on unrelated replicons. In this paper, we report that at least one RK2 function can act in *trans* to permit the replication and maintenance of a recircularized fragment of RK2 that contains the RK2 origin of replication. This origin-containing region is a defective replicon but can be maintained in the cell as a separate covalently closed circular (CCC) molecule in the presence of the cloned gene(s) for a *trans*-acting function(s). One component of the *trans* activity appears to be functioning negatively.

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## MATERIALS AND METHODS

**Bacterial and Phage Strains.** *E. coli* DF1020, a phage Mu lysogen of C600 (*leu thr thi lacY supE44 tonA*), has been described (8). C600 Δ*trpE5* and C600 Δ*trpE5 recA56* were provided by C. Yanofsky. DF1308 is a nalidixic acid-resistant (Nal$^r$) isolate of the latter strain. SR19 (*thr leu arg his pro thi thy recA13 lac gal* T6$^r$ *str xyl mtl ara supE*), C2107 (*his rha polA12*), and C2110 (*his rha polA1*) were provided by M. Kahn. W3110 *polA1* has been described (9). The phage PRR1 (10) and PRD1 (11) were obtained from R. Olsen.

**Plasmids.** pML31, pDF1, and pDF21 are mini-F hybrid plasmids constructed *in vitro*. pML31 is mini-F linked to an *Eco*RI DNA fragment coding for Km$^r$ (12). pDF1 and pDF21 are composed of mini-F linked to ColE1 (13) and *trpED* (14) *Eco*RI fragment, respectively (unpublished data). pDF37 is a hybrid between pML2 (13) and two tandem 5.2-kb fragments carrying *trpE* that have been inserted at the *Hin*dIII site of pML2 (unpublished data). One of the fragments resulting from cleavage of pDF37 with *Eco*RI is 5.2 kb and carries *trpE*. The following plasmids have been described: pSC101 (Tc$^r$) (15), RSF2124 (ColE1-Ap$^r$) (16), pRK212.1 (Ap$^r$ Tc$^r$ Km$^s$ Tra$^+$) and pRK214.1 (Ap$^r$ Tc$^r$ Km$^s$ Tra$^-$) (8), pCR1 (ColE1-Km$^r$) (17), and pRK256 (Fig. 1; unpublished data).

**Media and Reagents.** M9-CAA medium (used for selection and screening of Trp$^+$ strains), LB, and LB-glu media have been described (8). When required, antibiotics (Sigma) were added to the following concentrations: Km sulfate, 50 μg/ml; Tc hydrochloride, 30 μg/ml; penicillin (to select and screen for Ap$^r$), 150 μg/ml; Nal, 20 μg/ml. *Eco*RI was prepared by the procedure of Greene *et al.* (18). For labeling DNA, cells were grown in M9-CAA with [$^3$H]methylthymine [10 μCi/ml, 10 Ci/mmol; 1 Ci = 3.7 × $10^{10}$ becquerels; New England Nuclear] and 2 μg of thymine or M9-CAA per ml with [2-$^{14}$C]thymine (2 μg/ml, 50 mCi/mmol; New England Nuclear). *Hin*dIII and *Pst* I were purchased from New England BioLabs; T4 DNA ligase was from Miles.

**Procedures.** Preparation of plasmid DNA, reaction conditions for restriction endonucleases, transformation of *E. coli*, and spot testing for phage sensitivity have been described (8). Colicin sensitivity of bacterial strains was assayed according to Herschfield *et al.* (13). Reaction conditions for DNA ligation and the method for agarose gel electrophoresis are presented elsewhere (19).

For plasmid copy number determinations, 0.5 ml of a midlogarithmic phase culture (5 × $10^8$ cells/ml) grown in la-

Abbreviations: Ap, ampicillin; kb, kilobase(s); Km, kanamycin; Nal, nalidixic acid; Tc, tetracycline; $^r$, resistant; $^s$, sensitive; CCC, covalently closed circular.

* Present address: Department of Microbiology, College of Physicians and Surgeons, Columbia University, New York, NY 10032.

beling medium was lysed with Sarkosyl (8), and one-third of the lysate was centrifuged to equilibrium in a 5-ml CsCl/ethidium bromide density gradient with a Beckman 50 Ti rotor at 40,000 rpm for 40 hr at 15°C. Fractions were dripped onto filter paper and assayed for acid-precipitable $^3H$. To resolve the relative amounts of two plasmids, the remaining two-thirds of the lysate was centrifuged to equilibrium in a 7-ml CsCl/ethidium bromide gradient. Fractions were collected and assayed for $^3H$. Those fractions containing CCC DNA were pooled, dialyzed, precipitated, and resuspended (8). This DNA was mixed with $^{14}C$-labeled marker DNA, layered on a 5-ml 5–20% neutral sucrose gradient, and centrifuged in a Beckman SW 50.1 rotor at 45,000 rpm for 80 min at 15°C (19). Fractions were collected onto filter papers and assayed for acid-precipitable radioactivity. $^{14}C$ spillover into the $^3H$ channel was corrected for and the relative proportion of the plasmids was calculated as the fraction of total $^3H$ cpm in each plasmid band.

All recombinant DNA procedures were carried out in accordance with the National Institutes of Health Guidelines for Recombinant DNA Research.

## RESULTS

**Construction of Hybrid Plasmids Containing Regions Essential for Replication of RK2.** Two of the regions of RK2 thought to contain genes essential for replication in *E. coli* are the segments of 14–29.9 and 50.4–56.4 kb on the RK2 physical map (ref. 8; unpublished data). Both regions are present on the large *Eco*RI fragment A (41.7 kb) of the RK2 derivative pRK212.2 (Fig. 1). Fragment A specifies Km$^r$; *Eco*RI fragment B (14.4 kb) specifies Ap$^r$ and contains the RK2 origin of replication. Failure to transform *E. coli* for either Km$^r$ or Ap$^r$ using *Eco*RI-digested pRK212.2 DNA indicates that neither fragment A nor fragment B alone is able to replicate autonomously.

To maintain separately the essential genes on fragment A and the RK2 origin on fragment B, hybrid plasmids were constructed *in vitro* by using the ColE1 and mini-F replicons. A mixture of pRK212.2 and excess pDF1 (a ColE1–mini F hybrid described above) DNA was digested with *Eco*RI, incubated with T4 DNA ligase, and used to transform DF1020 for Km$^r$ or Ap$^r$. Plasmid DNA from one clone that was Km$^r$ Ap$^s$ and ColE1 immune was digested with *Eco*RI and analyzed by agarose gel electrophoresis. This plasmid (pRK2013) was found to contain the 6.3-kb ColE1 replicon and the 41.7-kb fragment A of pRK212.2. Another clone that was Km$^r$ Ap$^s$ but ColE1 sensitive carried a hybrid plasmid (pRK2023) composed of the 9-kb mini-F replicon and the pRK212.2 fragment A. Approximately 6% of the Km$^r$ colonies were Ap$^r$, and these were not characterized further. In no case did the transformation result in the generation of a Km$^s$ Ap$^r$ clone containing a hybrid of ColE1 or mini-F carrying only intact fragment B of pRK212.2.

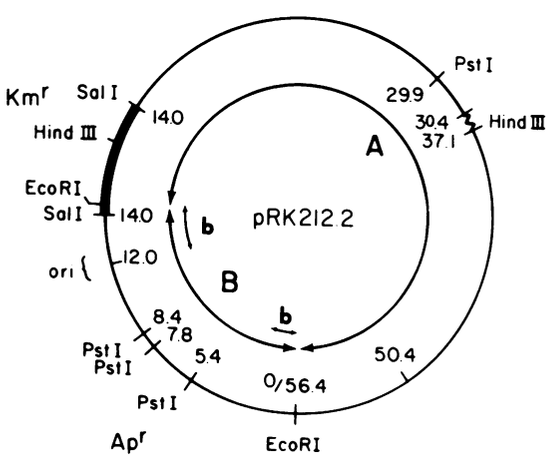


FIG. 1. Physical and genetic map of plasmid pRK212.2 (56.1 kb) constructed *in vitro* by the insertion of a 5.4-kb *Sal* I fragment (heavy line) that carries a Km$^r$ gene obtained from a mini-F-Km$^r$ plasmid, pML31 (12), into the single *Sal* I site of pRK212.1, a Km$^s$ deletion derivative of RK2 (8). The insertion disrupts the Tc$^r$ of RK2 but adds Km$^r$ and a new *Eco*RI site. "A" and "B" designate the fragments (arrows) generated by *Eco*RI cleavage. The wavy line indicates a segment (1 kb) of bacteriophage Mu DNA present in pRK212.1. The numbers refer to parental RK2 coordinates, defined as the distance in kilobases clockwise from the RK2 *Eco*RI site (6). Ori, origin of replication (7); b, locations of the 2.4-kb region present in pRK256 (unpublished observations).

Both pRK2013 and pRK2023 are self-transmissible. The frequency of pRK2013 transfer is comparable to that of RK2, whereas pRK2023 transfer is no more than 1/100th of it. Both plasmids are sensitive to the Inc P-1 specific phages PRR1 and PRD1, as determined by spot-testing. A W3110 *polA1* strain could be transformed with pRK2023 but not with pRK2013. This provides additional evidence that pRK212.2 fragment A will replicate only when it is linked to another replicon, because dependence on *E. coli* DNA polymerase I is a property of plasmid ColE1 (9) and not RK2 (unpublished data).

***trans*-Complementation of the Origin-Containing Fragment.** To determine if a hybrid plasmid containing pRK212.2 fragment A that carries an essential RK2 gene(s) would support the replication of the pRK212.2 fragment B region that contains the RK2 replication origin, the experiment described in Fig. 2 was carried out. pRK212.2 was digested with *Eco*RI and *Hin*dIII to generate *Eco*RI fragment B and cleave fragment A into smaller fragments. This mixture was used to transform strain DF2023 that carries the pRK2023 hybrid plasmid, and Ap$^r$ colonies were selected. Plasmid DNA from eight clones was prepared and characterized on agarose gels. Four of the eight clones examined carried two plasmids of different sizes. DNA from one of these clones, designated DF2067, is shown in Fig. 3, lane B. The other four clones contained only one plasmid with a molecular weight greater than that of pRK2023. The transformants that contained two plasmids were characterized further. Digestion of the two plasmids with *Hin*dIII resulted in the cleavage of only the large plasmid and gave the same pattern as did cleavage of pRK2023 (Fig. 3, lanes C and D). The smallest fragment obtained results from the *Hin*dIII cleavage site in the mini-F portion of pRK2023 (20, 21). However, both plasmids were cleaved with *Eco*RI (Fig. 3, lanes E and F). One

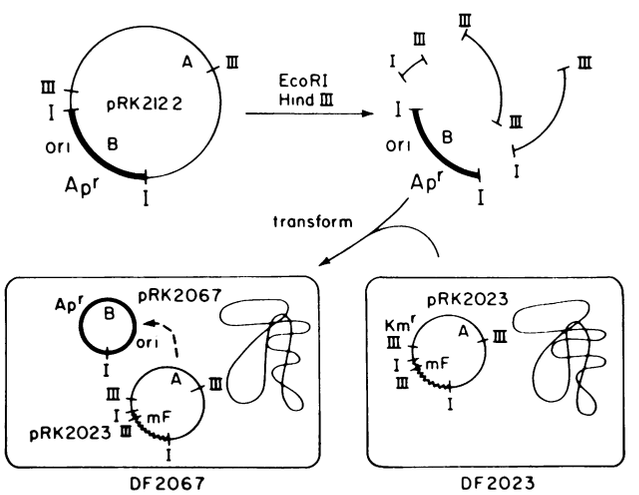


FIG. 2. Scheme for selection of the *trans*-complemented plasmid pRK2067. I and III, cleavage sites for *Eco*RI and *Hin*dIII, respectively; mF, mini-F replicon; other symbols as in Fig. 1. Experimental details are provided in the text.

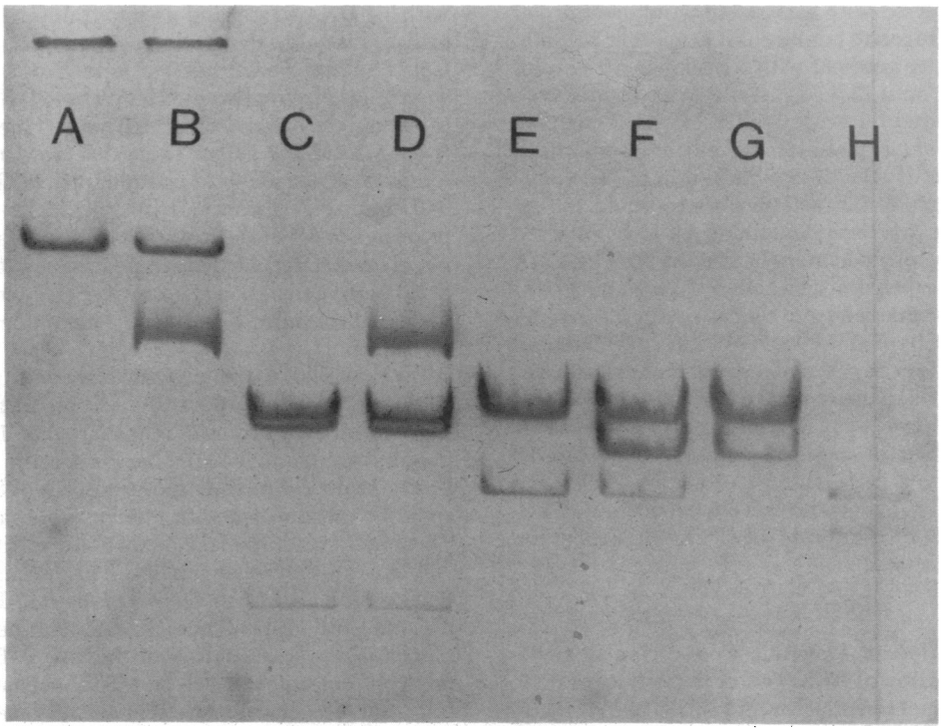


FIG. 3. Agarose gel (0.8%) analysis of DF2067 DNA. Lanes: A, undigested pRK2023; B, undigested plasmid DNA from DF2067; C, *Hin*dIII-digested pRK2023; D, *Hin*dIII-digested plasmid DNA from DF2067; E–H, *Eco*RI-digested pRK2023 (E), plasmid DNA from DF2067 (F), pRK212.1 (G), and pDF1 (H). In lane H, the upper (9 kb) band corresponds to mini-F and the lower (6.3 kb) band corresponds to ColE1.

fragment comigrated with pRK212.2 fragment B; the other two fragments comigrated with fragment A and the mini-F replicon from pRK2023. The intensity of the 14.4-kb band (fragment B) was greater than expected if it were equimolar with the other fragments; it was consistent with it being derived from a plasmid with a higher copy number. The circular DNA form of the small plasmid remaining after digestion of the two plasmids in DF2067 with *Hin*dIII was repurified in a CsCl/ethidium bromide density gradient. When this plasmid was digested with *Eco*RI, it generated only a single 14.4-kb fragment that comigrated with pRK212.2 fragment B (data not shown). This plasmid is designated pRK2067.

To verify that pRK2067 carried the $Ap^r$ gene that had been localized on fragment B of pRK212.2 and that replication of this plasmid was being complemented in *trans*, the following experiments were carried out. DF1020 strains with and without plasmid pML31 (mini-F $Km^r$) or pRK2023 were transformed with plasmid DNA from DF2067 (Table 1). In the case of transformation of the DF1020 strain that did not contain a plasmid, 54% of $Km^r$ colonies were also $Ap^r$ whereas 100% of the $Ap^r$ colonies were $Km^r$. The $Ap^r$ $Km^r$ colonies were found to contain two plasmids. Thus, $Km^r$ and $Ap^r$ are not linked on the same plasmid molecule and the recovery of $Ap^r$ depends on transformation with both plasmid elements. When pML31 was present in DF1020, the frequency of $Ap^r$ colonies decreased, presumably because transformation with pRK2023 was decreased due to incompatibility with the resident mini-F plasmid. As expected, the highest frequency of $Ap^r$ transformants was obtained with the DF1020 strain that carried the pRK2023 plasmid.

Treatment of the plasmid DNA of DF2067 with *Hin*dIII, which cleaves pRK2023 but not pRK2067, resulted in no $Ap^r$ transformants from DF1020 or DF1020 (pML31) (Table 1). In contrast, no significant decrease in transformation with the treated DNA was observed when DF1020 (pRK2023) was used as the recipient strain.

On the basis of these physical and genetic criteria, it is concluded that pRK2067 consists of pRK212.2 fragment B DNA alone. In addition, pRK2067 replication or maintenance is dependent upon a *trans*-acting function(s) encoded by pRK212.2 fragment A that is present on the pRK2023 helper plasmid.

A helper-dependent plasmid with a different selective marker also was constructed to facilitate analysis of the incompatibility properties of this RK2 derivative. Transformation of C600 *ΔtrpE5recA56* (pRK2023) with a mixture of *Eco*RI-cleaved pRK2067 and pDF37 (a ColE1-*trp* plasmid described in *Materials and Methods*) DNA that had been incubated with ligase resulted in $Trp^+$ $Ap^r$ clones that contained a hybrid pRK2067–Trp plasmid. One of these plasmids, pRK2076, was cleaved with *Pst* I to remove the region of DNA carrying the $Ap^r$ gene. Transformation with this cleaved DNA and selection for $Trp^+$ yielded pRK2080, a 16.6-kb plasmid that is $Trp^+$ $Ap^s$ and is dependent upon the pRK2013 or pRK2023 helper plasmid for its replication.

Table 1. Effect of pRK2023 on transformation frequency of pRK2067 DNA*

| Recipient strain | $Ap^r$ transformants, no. DF2067 CCC DNA | $Ap^r$ transformants, no. *Hin*dIII-digested DF2067 DNA |
|---|---|---|
| DF1020 | $1.3 \times 10^3$ | 0 |
| DF1020 (pML31) | $5 \times 10^2$ | 0 |
| DF1020 (pRK2023) | $4.4 \times 10^4$ | $3.8 \times 10^4$ |

* The *E. coli* strains indicated were transformed with 0.5 μg of supercoiled or *Hin*dIII-digested DNA from DF2067. To normalize for differences in transformability, each strain was transformed with RSF2124 (ColE1-$Ap^r$) DNA. The values in the table have been normalized to DF1020. DF1020 (pRK2023) and DF1020 (pML31) were 0.67 and 0.93, respectively, as efficient as DF1020 for transformation with RSF2124.

Table 2. Plasmid copy number

| Strain | CCC DNA* | Relative proportion of plasmids† | Plasmid copies per chromosome‡ pRK2023 | pRK2067 |
|---|---|---|---|---|
| SR19 | 0 | — | 0 | 0 |
| SR19 (pRK2023) | 4.6 | — | 3.4 | 0 |
| SR19 (pRK2023, pRK2067) | 7.4 | 0.66 (pRK2023) 0.34 (pRK2067) | 3.6 | 6.6 |

* Calculated as ($^3$H cpm of CCC DNA/$^3$H cpm of remaining DNA) × 100.
† Determined by velocity sedimentation of $^3$H-labeled CCC DNA through a neutral sucrose gradient. $^{14}$C-Labeled pRK2023 and pRK2067 DNA were used as markers.
‡ Assumes 3750 kb for the *E. coli* chromosome (22), 50.7 kb for pRK2023, and 14.4 kb for pRK2067.

**Properties of the *trans*-Complemented Plasmids.** The plasmid copy number for strains carrying pRK2023 or both pRK2023 and pRK2067 was estimated by analytical CsCl/ethidium bromide density gradient centrifugation of Sarkosyl lysates followed by separation of the plasmids by velocity sedimentation of the CCC DNA through a neutral sucrose gradient. The results shown in Table 2 indicate that the copy number of pRK2023 is somewhat higher than expected for a plasmid replicating under mini-F control (12, 23). pRK2067 was found to be maintained at a copy number that was almost twice that of pRK2023, which is consistent with the observed higher intensity of the 14.4-kb band shown in Fig. 3, lane F. The copy number of pRK2067 is very similar to that found for the parental RK2 plasmid (unpublished data).

Plasmid incompatibility is the inability of closely related plasmids to be maintained together in the absence of selection. The incompatibility properties of pRK2023 and pRK2080 are shown in Table 3. pRK2023 is incompatible with mini-F-*trp* (pDF21) but is maintained stably in strains carrying the RK2 plasmid derivative pRK212.1 (8). Thus, the pRK212.2 *Eco*RI fragment A does not express the strong incompatibility typical of RK2. In contrast, there is strong incompatibility between pRK212.1 and pRK2080. Therefore, the *trans*-complemented plasmid that carries the RK2 origin of replication also expresses incompatibility with RK2. Whether this expression is also dependent on the presence of the helper plasmid is not known.

Table 3. Plasmid incompatibility*

| Unselected plasmid | Selected plasmid† | % loss of unselected plasmid |
|---|---|---|
| pRK212.1 | | 0 |
| pRK212.2 | | 0 |
| pRK2023 | | 0 |
| pDF21 | | 0 |
| pRK2080 | | 7 |
| pRK212.1 | pRK212.2 (Km) | 100 |
| pRK212.2 | pRK212.1 (Tc) | 88 |
| pRK212.2 | pDF21 (Trp) | 0 |
| pDF21 | pRK212.2 (Km) | 0 |
| pRK2023 | pRK212.1 (Tc) | 0 |
| pRK212.1 | pRK2023 (Km) | 0 |
| pRK2023 | pDF21 (Trp) | 100 |
| pDF21 | pRK2023 (Km) | 100 |
| pRK2080 | pRK212.1 (Tc) | 100 |
| pRK212.1 | pRK2080 (Trp) | 100 |

* C600 Δ*trpE5 recA56* strains carrying the various combinations of plasmids were prepared by transformation and selection for both plasmids; 45 colonies from each strain were stabbed into plates containing media selective for only one of the plasmids. The colonies were picked and stabbed again into the same selective media for two more passages, after which each of the colonies was tested for the presence of the unselected plasmid.
† The phenotype selected is indicated in parentheses.

The ability of the defective pRK2067 and pRK2080 to be mobilized conjugally to recipient cells by the self-transmissible, helper plasmid pRK2013 was tested (Table 4). pRK2013 can mobilize and promote conjugal transfer of the unrelated plasmid pSC101 (15) but it did not promote transfer of pRK2067 or pRK2080 to either a recipient that carried pRK2023 or a recipient that lacked this helper plasmid. F′*lac* (Inc FI) and R64*drd*11 (Inc I) also were unable to mobilize the defective plasmids.

**Nature of the *trans*-Acting Function(s).** The failure to clone *Eco*RI fragment B of pRK212.2 on a stable replicon suggests that perhaps some function of the helper is acting negatively. To test this possibility, temperature-sensitive DNA polymerase I strains were constructed that contained both pRK2013 and pRK2067. Because pRK2013 is DNA polymerase I dependent, shifting this strain to high temperature results in loss of pRK2013 from the dividing cells. If pRK2013 is controlling the expression of potentially detrimental genes on pRK2067, then loss of pRK2013 might be expected to prevent further growth of the cell even in the absence of selection for either plasmid. The results of plating cultures at 42°C were consistent with this possibility. The presence of pRK2067 in the C2107 (pRK2013) strain caused loss of colony-forming ability at 42°C but not at 32°C. In contrast, pRK2067 had no effect on temperature-sensitive *polA* strains carrying pRK2023 at 42°C because the mini-F hybrid pRK2023 was not lost at high temperature. These results suggest the the pRK212.2 fragment A region on the helper plasmids provides a function that inhibits a lethal activity specified by pRK2067.

Because this lethal activity specified by fragment B prevents a direct test of whether or not fragment A is providing a *trans*-acting function(s) that is required for replication, the replication properties of a segment of fragment B that lacks this lethal property were examined. It has been demonstrated that a small segment of RK2 that contains the origin of replication

Table 4. Mobilization of *trans*-complemented plasmids

| Plasmids in the donor cells | Transmission frequency in recipients, transconjugants/donor DF1308 | DF1308 (pRK2023) |
|---|---|---|
| RK2 | 0.51 (Km) | 0.015 (Tc) |
| pRK2013 | 0.39 (Km) | |
| pRK2013, pSC101 | 0.81 (Tc) | |
| pRK2013, pRK2067 | $<7 \times 10^{-5}$ (Ap) | $<2.2 \times 10^{-4}$ (Ap) |
| pRK2013, pRK2080 | $<1.2 \times 10^{-7}$ (Trp) | $<5.3 \times 10^{-7}$ (Trp) |

Overnight cultures grown under selective conditions were washed once with LB-glu, diluted 1:10 in LB-glu, and incubated at 37°C (shaking) for 2.5 hr. The matings were done with a donor-to-recipient ratio of 1:10 with incubation at 37°C overnight (no shaking); the cultures then were plated on selective medium for transconjugants. Nal was used to select against the donor, which was always C600 Δ*trpE5recA56*. The selection for the transferred plasmid is indicated in parentheses.

Table 5. *trans*-complementation of pRK256*

| Recipient strain | *polA* | Km$^r$ transformants, no./ml pRK256 DNA | Km$^r$ transformants, no./ml pCR1 DNA |
|---|---|---|---|
| C2110 | − | 0 | 0 |
| C2110 (pRK2045) | − | $3.6 \times 10^5$ | $1.8 \times 10^3$† |
| C600 Δ*trpE5* | + | $2.9 \times 10^5$ | $2.4 \times 10^5$ |
| C600 Δ*trpE5* (*pRK2045*) | + | $3.4 \times 10^5$ | $3.1 \times 10^5$ |

* To normalize for differences in transformability, each strain was transformed with PRK212.1 DNA. The values in the table are corrected values.

† Colonies showed large variation in size, ranging from pinpoint to normal. Because the Km$^r$ fragment present in pCR1 and pRK256 is also present in a Km$^s$ form in pRK2045, some background due to recombination of the plasmids is expected.

(Fig. 1) (R. Meyer and C. Thomas, personal communication) can be cloned on pCR1 [a ColE1 Km$^r$ plasmid (17)]. This hybrid plasmid, designated pRK256, shows none of the cell-killing activity exhibited by pRK2067. Because pRK256 will not replicate in an *E. coli polA* mutant, it is possible to test directly whether or not a fragment A-containing hybrid plasmid can provide a function to replicate pRK256 under RK2 control. Because pRK256 is Km$^r$, a Km$^s$Tc$^r$Trp$^+$ helper plasmid (pRK2045) was used. pRK2045 is a hybrid of pSC101 and pRK212.2 fragment A linked at their *Eco*RI cleavage sites. The Km$^r$ gene is interrupted by an insertion of a *trpE* fragment at the *Hin*dIII site. The results (Table 5) demonstrate that pRK256 will transform a *polA* host only when the pRK2045 helper plasmid is present. In the case of all transformants examined, Km$^r$ is specified by a separate plasmid. This result indicates that a 2.4-kb origin region of RK2 provides sufficient information for replication of the pRK256 plasmid when a *trans*-acting function is provided by the fragment A-containing helper plasmid.

## DISCUSSION

These results demonstrate that at least one essential gene of RK2 codes for a product that can act in *trans* to permit the replication of a segment of DNA carrying the RK2 origin of replication. The *trans*-complemented plasmid exists in the cell as a CCC DNA molecule that is physically separate from, but functionally dependent on, the presence of another replicon carrying the *trans*-acting RK2 gene(s).

The inability to obtain deletions of the 14 to 33.2-kb region (6) or the 50.4 to 56.4-kb region (8) of RK2 suggests that genes essential for RK2 replication or maintenance in *E. coli* are localized within both of these segments. Because the helper plasmids constructed in this work contained both segments, the results verify directly that at least one of these two regions codes for a *trans*-acting essential function.

The *trans*-complemented defective plasmid pRK2067 is composed almost entirely of the 0 to 14-kb portion of RK2. From the results with pRK256, it appears that most of this region other than the origin of replication, located at approximately 12 kb on the RK2 genome map (ref. 7; unpublished data), is not essential for replication in *E. coli*. The simplest explanation for our results is that a *trans*-acting product interacts with the RK2 origin on the defective plasmid to promote its replication. It is also possible that other gene products necessary for replication and maintenance are coded for by this DNA segment as well and that these genes are regulated by the helper function(s).

The finding that the presence of the defective pRK2067 plasmid causes loss of colony-forming ability when the helper plasmid is removed suggests that the *trans*-complementing activity in this case also may involve negative regulation of a gene(s) that is otherwise toxic to the cell. Although the function and exact location of this gene(s) in the 0- to 14-kb segment are not known, it is of interest that plasmid RK2 contains noncontiguous genetic regions, other than those required for replication, that must interact for the maintenance of the plasmid under normal conditions.

Conjugal transfer of the defective plasmid is not promoted by self-transmissible plasmids. The requirement for a helper plasmid and failure to mobilize the *trans*-complemented plasmid with the helper plasmid are important biological containment features for the possible use of the defective plasmid as a cloning vehicle. In this regard, pRK2067 has single cleavage sites for the restriction endonucleases *Eco*RI, *Sal* I, *Bam*HI, and *Bgl* II in regions not essential for replication. Hybrids constructed with this plasmid would be strictly dependent upon a specially constructed host strain carrying the helper functions on another plasmid or perhaps integrated into the chromosome.

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