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Robert L. Burghoff

David C. Laux
University of Rhode Island, dlaux@uri.edu

Paul S. Cohen
University of Rhode Island, pscohen@uri.edu

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Construction of Stable Cloning Vectors That Do Not Segregate from a Human Fecal *Escherichia coli* Strain in the Streptomycin-Treated Mouse Large Intestine

ROBERT L. BURGHOFF, DAVID C. LAUX, AND PAUL S. COHEN*

Department of Microbiology, University of Rhode Island, Kingston, Rhode Island 02881

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***Escherichia coli* F-18 Col$^-$ was previously shown to be a poor colonizer of the streptomycin-treated mouse large intestine, relative to its parent, *E. coli* F-18. Prior to attempting to clone genes responsible for the colonization phenotype of *E. coli* F-18 into *E. coli* F-18 Col$^-$, a suitable cloning vector had to be found. In this investigation, we report that the commonly used cloning vectors pBR322, pHC79, and pBR329 all segregate from *E. coli* F-18 Col$^-$ both when grown in L broth under conditions of nonselection (i.e., in vitro) and when fed to streptomycin-treated mice (i.e., in vivo). Insertion of the *cer* region (which promotes resolution of replicating plasmids into monomeric forms) into pHC79 stabilized this plasmid in *E. coli* F-18 Col$^-$ in vitro and in vivo. In contrast, two independent *cer* insertions into pBR329 did not stabilize the plasmid completely in *E. coli* F-18 Col$^-$ in vitro, and feeding the strain to streptomycin-treated mice resulted in rapid segregation of the plasmids in vivo. Also, stability of all three plasmids in *E. coli* F-18 Col$^-$ in vitro was achieved by insertion of the *parB* region of plasmid R1, which encodes a cell-killing protein, Hok, that is active only postsegregationally. However, as with *cer*, complete in vitro and in vivo stabilization was achieved only in *parB* constructs of pBR322 and pHC79.**

Escherichia coli F-18, isolated from the feces of a healthy person in 1977, is an excellent colonizer of the streptomycin-treated mouse large intestine (5). *E. coli* F-18 Col$^-$, a derivative of *E. coli* F-18, colonizes the streptomycin-treated mouse large intestine as well as its parent does when either is fed to mice alone, but it is unable to colonize when fed to mice together with the parent strain because of the inability of *E. coli* F-18 Col$^-$ to grow in cecal mucus in the presence of *E. coli* F-18 (20). Furthermore, when streptomycin-treated mice are simultaneously fed 10^8 CFU of *E. coli* F-18 Col$^-$ and only 10^3 CFU of *E. coli* F-18, *E. coli* F-18 becomes the predominant strain in the mouse large intestine within a few days (20). These data suggested the possibility of shotgun-cloning genes from *E. coli* F-18 into *E. coli* F-18 Col$^-$ and the subsequent selection by the mouse intestine of those clones that contain the functional *E. coli* F-18 colonization gene(s) that are defective or missing in *E. coli* F-18 Col$^-$. However, prior to cloning, a suitable highly stable vector (i.e., one which would not segregate in vivo) had to be chosen to carry these putative colonization genes. Here we present evidence that several commonly used cloning vectors are unstable in *E. coli* F-18 Col$^-$ both in vitro and in vivo and that insertion of either of the stabilizing loci *cer* or *parB* into these vectors can enhance the stability of these plasmids significantly.

MATERIALS AND METHODS

Bacterial strains. A spontaneous double mutant of *E. coli* HB101 (4) resistant to both streptomycin sulfate (Str^r) and nalidixic acid (Nal^r) was used for the isolation and characterization of relevant constructions. *E. coli* F-18 Col$^-$, used for all plasmid stability assays reported here, was originally isolated (5) as follows. A streptomycin-resistant, nalidixic acid-resistant mutant of *E. coli* F-18 was mated with *E. coli* RS2, which contains the R1drd19 plasmid (R1$^+$), and an *E. coli* F-18 R1$^+$ strain was isolated. The *E. coli* F-18 R1$^+$ strain was cured of the R1drd19 plasmid with acriflavin. One of the cured clones, *E. coli* F-18 Col$^-$, lost both a 3×10^7-dalton plasmid and the ability to make the F-18 colicin (5).

Media. The bacterial growth medium for all strains was L broth (2). The plating medium for both the in vitro and in vivo stability assays was MacConkey agar (Difco Laboratories, Detroit, Mich.) with additional antibiotics as needed: streptomycin sulfate, nalidixic acid, tetracycline hydrochloride, kanamycin monosulfate, ampicillin, and chloramphenicol at concentrations of 100, 50, 10, 40, 100, and 40 μg/ml, respectively.

Bacterial transformation. Both *E. coli* HB101 (Str^r Nal^r) and *E. coli* F-18 Col$^-$ (Str^r Nal^r) were made competent and transformed with plasmid DNA by the method of Mandel and Higa (14).

Biochemical and recombinant DNA techniques. Small-scale preparations of plasmid DNA were done by the method of Birnboim and Doly (3). Agarose gel electrophoretic analysis of restriction fragments was carried out with 1.5% agarose for detection of fragments of less than 500 base pairs and 0.8% agarose for all other applications. Ligation and restriction endonuclease reactions were done essentially as described by the manufacturer (Bethesda Research Laboratories, Gaithersburg, Md.). Calf intestinal phosphatase treatments were done essentially as described by the manufacturer (Boehringer Mannheim Biochemicals, Indianapolis, Ind.), and all restriction fragment purifications were performed with GENECLEAN according to the instructions provided by the manufacturer (Bio 101 Inc., La Jolla, Calif.).

Plasmid constructions. Plasmids used in this study are listed in Table 1. pRLB1 is a cosmid construct originating from a *Bam*HI-*Hin*dIII digest of pKS492 (18). The resulting 280-base-pair fragment, containing *cer*, was treated with GENECLEAN and ligated to a phosphatase-treated *Bam*HI-*Hin*dIII digest of pHC79 (12). pRLB2 is a cosmid construct derived from an *Eco*RI-*Bam*HI digest of pPR633 (9), which

* Corresponding author.

TABLE 1. Properties of plasmids used and constructed

Plasmid	Replicon	Characteristics[a]	Location[b]	Reference
pBR322	pMB1	*bla tet*	–	1
pHC79	pBR322	*bla tet* cos	–	12
pBR329	pBR322	*bla tet cat*	–	6
pJBK5	ColE1	*tet cat*	–	13
pPR633	pBR322	*bla parB*	0 to +580	11
pRLB1	pBR322	*bla cer*	+30 to +328	This article
pRLB2	pBR322	*bla parB*	0 to +598	This article
pRLB3	pBR322	*bla tet cer*	+102 to +1,102	This article
pRLB4	pBR322	*bla tet cer*	+102 to +1,102	This article
pRLB5	ColE1	*tet cat*	–	This article
pRLB6	pBR322	*tet cat aphA parB*	+2,750 to +4,380	This article

[a] *aphA*, Kanamycin resistance; *bla*, ampicillin resistance; *tet*, tetracycline resistance; *cat*, chloramphenicol resistance; cos, cohesive ends.
[b] Location of stability determinant, in base pairs, for all constructions. For all plasmids for which a coordinate location is given, the reference point (0 base pair) is the *Eco*RI site.

produces a 580-base-pair fragment containing *parB*, and subsequently ligated to analogously cleaved and phosphatase-treated pHC79 molecules. pRLB3 and pRLB4 represent two promoter orientations of ColE1 resolution containing pBR329 derivatives. These plasmids were generated by cloning a 1.0-kilobase (kb) *Pvu*II fragment of pKS492 containing *cer* into the single *Pvu*II site of pBR329 (6). pRLB5 was constructed from a GENECLEAN-purified 1.0-kb fragment of an *Mlu*I digest of lambda DNA cloned into the single phosphatase-treated *Mlu*I site of pJBK5, presumably interrupting *cer* function (13). pRLB6 is a kanamycin monosulfate-resistant pBR329 derivative constructed by cloning the 1.6-kb *Pst*I fragment of pKG1022, containing *parB* and the *aphA* gene (8), into the single *Pst*I site of pBR329.

In vivo segregation assays. The method for in vivo segregations was the same as that used to distinguish between the relative colonizing abilities of *E. coli* strains in mice and was described in detail previously (5, 20). Briefly, three 5- to 8-week-old CD-1 mice were given drinking water containing 5 g of streptomycin sulfate per liter. After 1 day, the mice were starved for 16 to 24 h for water and food (Charles River Valley Rat, Mouse and Hamster Formula) and then fed 10^{10} CFU of an L broth-grown, plasmid-containing *E. coli* F-18 Col$^-$ strain suspended in 1 ml of sterile 20% (wt/vol) sucrose solution. After consuming the bacterial suspension, the mice were returned to a diet of food and streptomycin-containing water. The following day and at 48-h intervals thereafter, 1 g of feces no older than 24 h from each of three mice was collected, suspended, homogenized, diluted, and plated on selective media to differentiate between plasmid-bearing and plasmid-free cells, as described previously (5). In all experiments reported here, plates were incubated at 37°C for 16 to 24 h. Plasmid stability was assessed by the level at which plasmid-bearing cells of strain *E. coli* F-18 Col$^-$ persisted in feces. Assays were performed at least twice, with essentially identical results.

In vitro segregation assays. Initially, plasmid-containing *E. coli* F-18 Col$^-$ cells were inoculated into tubes containing 5 ml of L broth with either 100 μg of ampicillin per ml or 10 μg of tetracycline per ml, as required. Cultures were grown to stationary phase (standing) and serially diluted 10^6-fold into 5 ml of L broth (no antibiotics added). This dilution, representing approximately 10^3 CFU/ml, was regrown overnight at 37°C, serially diluted, and plated onto selective media to distinguish between plasmid-containing and plasmid-free cells. Sequential 10^6-fold dilutions were regrown to stationary phase every 24 h for a total of 11 days. Each 24-h cycle represents approximately 20 generations of growth, for a total of about 220 generations per strain.

Statistics. Where indicated in the text, means derived from triplicate samples were compared by using Student's *t* test (*P* values).

RESULTS

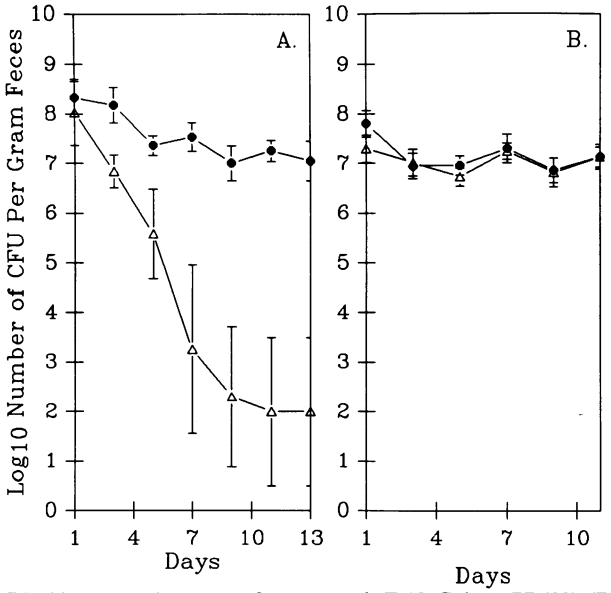
pBR322. pBR322, a 4.3-kb, ColE1-relaxed replicon, is a standard cloning vector used in numerous cloning and expression applications in molecular biology (1). pBR322 is well documented, and its entire nucleotide sequence has been elucidated (19). The plasmid has a copy number of about 18 per genome under steady-state conditions in *E. coli* K-12 strains (7). pBR322 segregated readily from *E. coli* F-18 Col$^-$ both in vitro, so that after about 220 generations only about 1 of 600 cells contained the plasmid (Table 2), and in vivo, so that by day 7 postfeeding only about 1 of 10^4 cells contained the plasmid (Fig. 1A). However, pPR633, which contains *parB* inserted into pBR322 (9), was completely stable in *E. coli* F-18 Col$^-$ both in vitro (Table 2) and in vivo (Fig. 1B).

pHC79. pHC79 is a cosmid derivative of pBR322 made from a pBR322 *Sau*I-induced rearrangement, which has a single *Bgl*II site (pJC80, 2.95 megadaltons) located between the end of the tetracycline gene and the origin of replication. A 1.1-megadalton *Bgl*II fragment containing the cos site

TABLE 2. In vitro plasmid segregation from *E. coli* F-18 Col$^-$

Plasmid	Ratio of total CFU/ml to plasmid-containing CFU/ml after:		
	20 generations	140 generations	220 generations
pBR322	1.02	39.5	618
pPR633	1.04	1.02	1.02
pHC79	1.59	88.9	1,778
pRLB1	0.91	1.14	0.91
pRLB2	0.89	0.89	1.00
pBR329	1.07	11.90	105
pRLB3	0.83	1.25	3.38
pRLB4	1.14	1.19	3.39
pRLB6	1.25	0.98	3.25[a]

[a] This ratio was confirmed by transferring (with toothpicks) 55 colonies from plates containing streptomycin and nalidixic acid (total CFU plates) to plates containing streptomycin, nalidixic acid, and kanamycin in order to score pRLB6-containing (kanamycin-resistant) CFU and pRLB6-free (kanamycin-sensitive) CFU.



(pBR322) (A) and pPR633 from *E. coli* F-18 Col$^-$ (pPR633) (B). Symbols: ●, total CFU; △, plasmid-containing CFU.

from lambda Charon 3A was cloned into that unique site, allowing for in vitro packaging of large sequences of foreign DNA into lambda bacteriophage capsids (12). pHC79 behaved similarly to pBR322 in that pHC79 segregated rapidly from *E. coli* F-18 Col$^-$ both in in vitro (Table 2) and in vivo (Fig. 2A). Insertion of either *cer*, to yield pRLB1, or *parB*, to yield pRLB2, completely stabilized this plasmid in *E. coli* F-18 Col$^-$ both in vitro (Table 2) and in vivo (Fig. 2B and C).

pBR329. pBR329 is a high-copy number variant of pBR322, i.e., 30 copies per genome in *E. coli* K-12 strains (15). The plasmid is a 4.15-kb hybrid construct of pBR327 and pBR328 made from an *Hha*I digest of pBR328 (carrying the chloramphenicol resistance gene) treated with *Bal*I exonuclease. The resulting fragments were ligated to an S1 nuclease-treated *Eco*RI site of pBR327 (6). pBR329 segregated from *E. coli* F-18 Col$^-$ both in vitro (Table 2) and in vivo (Fig. 3A). Although two independent *cer* insertions (to yield pBRL3 and pBRL4) significantly stabilized pBR329 in vitro (Table 2), neither construct had an effect on the rapid rate of segregation in vivo (Fig. 3B; one construct, pBRL3, is shown). Similarly, insertion of the *parB* region of plasmid R1 into pBR329 (to yield pBRL6) stabilized pBR329 to a great extent in *E. coli* F-18 Col$^-$ in vitro (Table 2); however, in vivo (Fig. 3C), the addition of *parB* still resulted in plasmid segregation.

pJBK5. During the course of this work, pJBK5, a ColE1 derivative (13), was tested in *E. coli* F-18 Col$^-$ for its segregation rate in vivo and was found to be stable (Fig. 4A). Since the original ColE1 plasmid contains the *cer* region, which has a unique *Mlu*I site (18), pJBK5 was restricted with *Mlu*I and a small piece of bacteriophage lambda DNA was inserted to disrupt the *cer* function (see Materials and Methods). The resulting construct, pRLB5, was subsequently transformed into *E. coli* F-18 Col$^-$ and found to segregate rapidly in vivo (Fig. 4B). These data indicate that pJBK5 stabilization is most likely caused by *cer* function.

DISCUSSION

The commonly used cloning vectors pBR322, pHC79, and pBR329 were all found to be unstable in *E. coli* F-18 Col$^-$ both in vitro and in vivo (Table 2 and Fig. 1A, 2A, and 3A). Insertion of the *cer* region, a site-specific recombination sequence which promotes conversion of plasmid multimers (which occur through *recA*-dependent homologous recombination) to monomers, in conjunction with the *trans*-acting chromosomally encoded *xerA* gene product (16), has been shown previously to stabilize plasmids in vitro (17). In our hands, *cer* prevented segregation of pHC79 completely both in vitro and in vivo (Table 2 and Fig. 2B). A pBR322 *cer* plasmid was not constructed because it is closely related to pHC79. Two distinct *cer* promoter orientation constructions of pBR329 stabilized the plasmid in vitro; however, between generations 140 and 220, segregation in vitro was observed (Table 2). Both constructs segregated rapidly in vivo (one is shown in Fig. 3B).

As was observed for *cer*, the *parB* region of plasmid R1 stabilized the plasmid in vitro i.e., pBR322 and pHC79 were

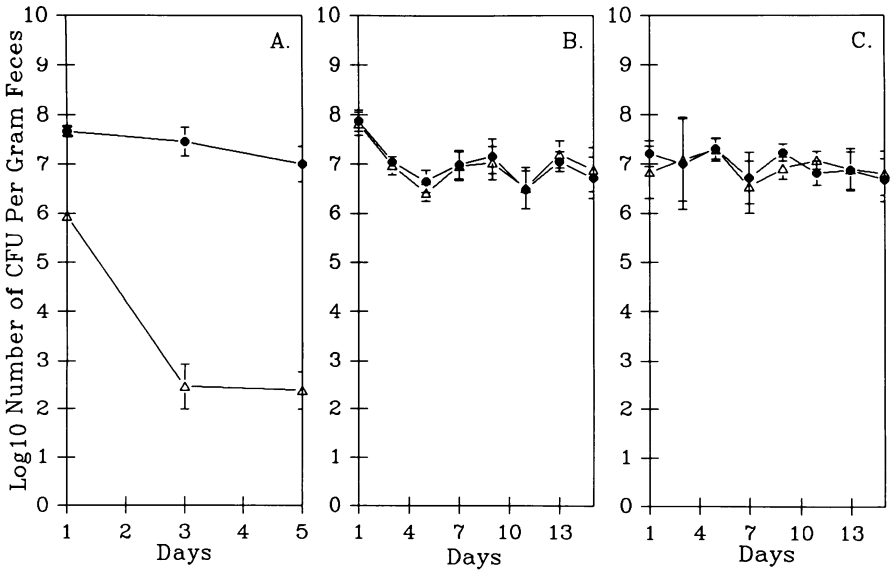


FIG. 2. In vivo segregation of pHC79 from *E. coli* F-18 Col$^-$ (pHC79) (A), pRLB1 from *E. coli* F-18 Col$^-$ (pRLB1) (B), and pRLB2 from *E. coli* F-18 Col$^-$ (pRLB2) (C). Symbols: ●, total CFU; △, plasmid-containing CFU.

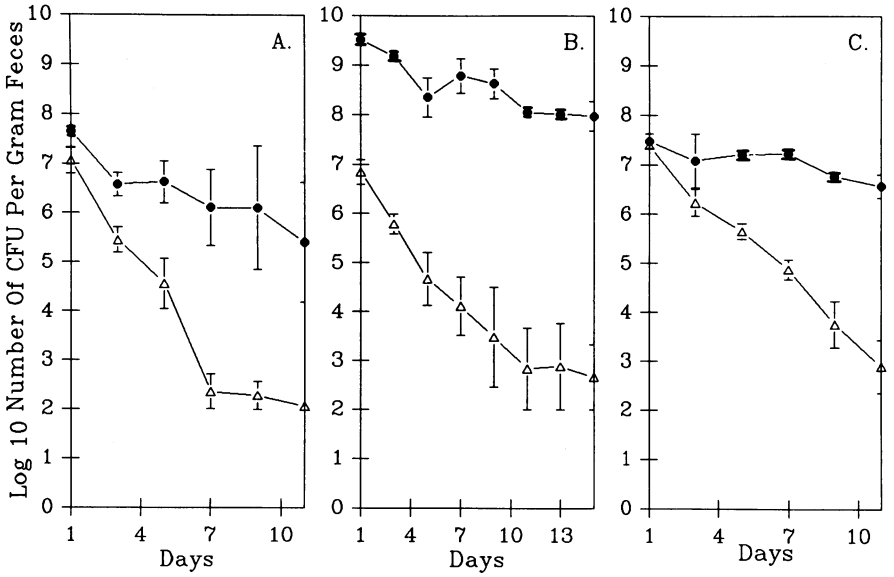


FIG. 3. In vivo segregation of pBR329 from *E. coli* F-18 Col$^-$ (pBR329) (A), pRLB3 from *E. coli* F-18 Col$^-$ (pRLB3) (B), and pRLB6 from *E. coli* F-18 Col$^-$ (pRLB6) (C). Symbols: ●, total CFU; △, plasmid-containing CFU.

stabilized completely, and pBR329 showed reduced segregation in *E. coli* F-18 Col$^-$ (Table 2). As with *cer*, *parB* stabilized pBR322 and pHC79 in vivo but was unable to stabilize pBR329 in vivo (Fig. 3C). This is somewhat surprising, since segregation of a plasmid containing the *parB* region is supposed to result in postsegregational killing of the plasmid-free cell (11). That is, the cell-killing gene *hok*, located within the *parB* region, encodes a 52-amino-acid protein which causes collapse of transmembrane potential (9). In plasmid-containing cells, *hok* expression is prevented by an antisense RNA, *sok*, which is also encoded within the *parB* region. The antisense RNA forms an RNA-RNA hybrid with the *hok* transcript inhibiting translation. Segregation of a *parB*-containing plasmid leads to a rapid degradation of *sok* RNA, leaving *hok* mRNA free for translation of the 52-amino-acid product, which kills the plasmid-free cells (10). Therefore, the *parB* region ensures that all live bacterial cells contain the plasmid. Apparently, some *E. coli* F-18 Col$^-$ cells which segregate the pBR329 derivative containing *parB* escape death in vivo and efficiently colonize the streptomycin-treated mouse large intestine. Although we do not understand why pBR329 containing *cer* and *parB* segregates slowly in vitro and rapidly in vivo, the fact that it does underscores the idea that even the most stable plasmids in vitro should be tested for segregational stability in vivo. In any case, plasmids pPR633, pRLB1, and pRLB2 are stable constructs and seem ideally suited for in vivo selection of recombinant clones containing colonization genes.

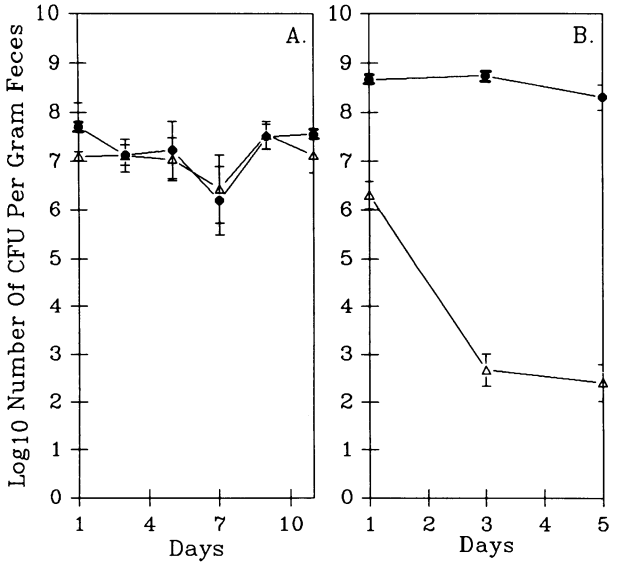


FIG. 4. In vivo segregation of pJBK5 from *E. coli* F-18 Col$^-$ (pJBK5) (A) and pRLB5 from *E. coli* F-18 Col$^-$ (pRLB5) (B). Symbols: ●, total CFU; △, plasmid-containing CFU.

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