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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

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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 10⁸ CFU of E. coli F-18 Col - and only 10⁹ 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 (StrR) and nalidixic acid (NalR) 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 R1дрd19 plasmid (R1 +), and an E. coli F-18 R1 + strain was isolated. The E. coli F-18 R1 + strain was cured of the R1дрd19 plasmid with acriflavin. One of the cured clones, E. coli F-18 Col -, lost both a 3 × 10⁷-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 (StrR NalR) and E. coli F-18 Col - (StrR NalR) 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 done 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 BamHI-HindIII digest of pKS492 (18). The resulting 280-base-pair fragment, containing cer, was treated with GENECLEAN and ligated to a phosphatase-treated BamHI-HindIII digest of pHC79 (12). pRLB2 is a cosmid construct derived from an EcoRI-BamHI digest of pPR633 (9), which

* Corresponding author.
TABLE 1. Properties of plasmids used and constructed

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Replicon</th>
<th>Characteristics</th>
<th>Location</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBR322</td>
<td>pMB1</td>
<td>bla tet</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>pH79</td>
<td>pBR322</td>
<td>bla tet cos</td>
<td></td>
<td>12</td>
</tr>
<tr>
<td>pBR329</td>
<td>pBR322</td>
<td>bla tet cat</td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>pJBK5</td>
<td>CoEI</td>
<td>tet cat</td>
<td></td>
<td>13</td>
</tr>
<tr>
<td>pPR633</td>
<td>pBR322</td>
<td>bla parB</td>
<td>0 to +580</td>
<td>11</td>
</tr>
<tr>
<td>pRLB1</td>
<td>pBR322</td>
<td>bla cer</td>
<td>+30 to +328</td>
<td>This article</td>
</tr>
<tr>
<td>pRLB2</td>
<td>pBR322</td>
<td>bla parB</td>
<td>0 to +598</td>
<td>This article</td>
</tr>
<tr>
<td>pRLB3</td>
<td>pBR322</td>
<td>bla tet cer</td>
<td>+102 to +1,102</td>
<td>This article</td>
</tr>
<tr>
<td>pRLB4</td>
<td>pBR322</td>
<td>bla tet cer</td>
<td>+102 to +1,102</td>
<td>This article</td>
</tr>
<tr>
<td>pRLB5</td>
<td>CoEI</td>
<td>tet cat</td>
<td></td>
<td>This article</td>
</tr>
<tr>
<td>pRLB6</td>
<td>pBR322</td>
<td>tet cat apmA parB</td>
<td>+2,750 to +4,380</td>
<td>This article</td>
</tr>
</tbody>
</table>

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 EcoRI site.

TABLE 2. In vitro plasmid segregation from E. coli F-18 Col–

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Ratio of total CFU/ml to plasmid-containing CFU/ml after:</th>
<th>20 generations</th>
<th>140 generations</th>
<th>220 generations</th>
</tr>
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<tbody>
<tr>
<td>pBR322</td>
<td>1.02</td>
<td>39.5</td>
<td>618</td>
<td></td>
</tr>
<tr>
<td>pPR633</td>
<td>1.04</td>
<td>1.02</td>
<td>1.02</td>
<td></td>
</tr>
<tr>
<td>pH79</td>
<td>1.59</td>
<td>88.9</td>
<td>1,778</td>
<td></td>
</tr>
<tr>
<td>pRLB1</td>
<td>0.91</td>
<td>1.34</td>
<td>0.91</td>
<td></td>
</tr>
<tr>
<td>pRLB2</td>
<td>0.89</td>
<td>0.89</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>pBR329</td>
<td>1.07</td>
<td>11.90</td>
<td>105</td>
<td></td>
</tr>
<tr>
<td>pRLB3</td>
<td>0.83</td>
<td>1.25</td>
<td>3.38</td>
<td></td>
</tr>
<tr>
<td>pRLB4</td>
<td>1.14</td>
<td>1.19</td>
<td>3.39</td>
<td></td>
</tr>
<tr>
<td>pRLB6</td>
<td>1.25</td>
<td>0.98</td>
<td>3.25</td>
<td></td>
</tr>
</tbody>
</table>

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.
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). pH79 behaved similarly to pBR322 in that pH79 segregated rapidly from E. coli F-18 Col^- both 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 Hhal digest of pBR328 (carrying the chloramphenicol resistance gene) treated with BalI exonuclease. The resulting fragments were ligated to an S1 nuclease-treated EcoRI 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 pRL3 and pRL4) significantly stabilized pBR329 in vitro (Table 2), neither construct had an effect on the rapid rate of segregation in vivo (Fig. 3B; one construct, pBR3, is shown). Similarly, insertion of the parB region of plasmid R1 into pBR329 (to yield pRL6) 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 MluI site (18), pJBK5 was restricted with MluI 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, pH79, 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 pH79 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 pH79. 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 pH79 were...
stabilized completely, and pBR329 showed reduced segregation in E. coli F-18 Col⁻ (Table 2). As with cer, parB stabilized pBR322 and pH79 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.

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LITERATURE CITED

6. Covarrubias, L., and F. Bolivar. 1982. Construction and char-

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.

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.


