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Molecular Cloning of the Region of the Terminus of *Escherichia coli* K-12 DNA Replication

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A series of plasmids have been isolated either by ligation of defined restriction fragments to plasmid pBR325 or by screening of a cosmid bank by in situ colony hybridization. Together with one previously isolated plasmid, they spanned 86% of the 30.5- to 34-min region of the genetic map of *Escherichia coli* K-12. Physical analysis of these plasmids and hybridizations to Southern blots confirmed the endonuclease map of this region, with the exception of a 9.3-kilobase pair inversion.

The region of the chromosome of *Escherichia coli* K-12 between the cryptic prophage *rac* (29.7 to 30.1 min) and the gene coding for the mannose phosphate isomerase *manA* (35.7 min) possesses two exceptional features. First, it contains a terminus of DNA replication, called *terC*. Evidence for the terminus was obtained by demonstrating that genetically engineered displacements of the functional origin of replication still result in DNA replication ending in this region (13–15). Second, the region of the terminus is remarkably devoid of characterized genes, particularly between *trg* (31.4 min) and *relB* (34.4 min). A schematic genetic map indicating the known genes of the *rac–manA* region is shown in Fig. 1. In addition to the genes shown in the map of Bachmann and Low (1), it includes *ksgD* at 30.4 min (8), the inaccurately mapped gene *rimL* (12), and *kim*, a defective prophage defined from its cross-hybridization with phage λ DNA (6).

Substantial progress in the analysis of this region has been recently reported. Using transposon insertions, Bitner and Kuempel (3) and Fouts and Barbour (9) independently closed the cotransduction gap between *rac* and *manA*. A restriction endonuclease map of the *trp* (27.5 min)–*manA* region has been constructed (5) and colinearized with the genetic map. The average point at which replication forks initiated from *oriC* encounter each other was found at 31.2 min in a *trp*::Mu lysogen (6). Therefore, the conditions are met for both fine genetic and physical analysis of the region of the terminus of DNA replication. We report here one step in this direction. Thanks to previous knowledge of the restriction map, we have undertaken the cloning of various segments located between *rac* and *manA*. The construction, screening, and physical analysis of these plasmids are reported in this paper.

MATERIALS AND METHODS

Bacterial strains. The *E. coli* K-12 strains used in this study were the following: CB0129 (W1485F⁻ *leu thyA deoB* or *-C*) (our collection), LN681 (F⁻ *thyA deoB* or *-C gyrA dnaC28 trpB*::Mu) (5), C600 (F⁻ *leu thi gyrA lacY thr tonA*), and HB101 (F⁻ *pro leu thi rpsL lacY hsdS endA recA*).

Preparation of size classes of chromosomal DNA fragments. DNA from strain LN681 labeled at the end of a synchronized replication cycle was prepared, digested, and fractionated as previously described (5). The radioactivity profile of the preparative gel was compared with a canonical profile of the gel from which the restriction map of the *trp–manA* region was constructed (5). Pools of extracted DNA were made, each containing fragments of a given size class selected for the probable presence of a fragment from the terminus region.

Unlabeled DNA from strain LN681 was also cut with restriction endonucleases and mixed with samples of the labeled pools. After electrophoretic fractionation, the DNA from slices containing label was extracted and used as a source of material to be cloned.

Ligation, transformation, and preparation of plasmids. DNA fragments were ligated to linearized DNA from plasmid pBR325 which had been previously treated with alkaline phosphatase (19). Upon transformation of strain C600 and selection for chloramphenicol resistance, 25 to 85% of the colonies obtained carried hybrid plasmids. Plasmid DNA was prepared for analytical and preparative purposes as previously described (6).

Colony hybridization. A pool of encapsidated hybrid cosmids was generously supplied by W. Lindenmaier and J. Collins. It was made from a *Sau*3A partial digest of *E. coli* K-12 DNA ligated at the *Bam*HI site of plasmid pHC79 (11). The pool was transduced into strain HB101. Ampicillin-resistant colonies (Amp^r)

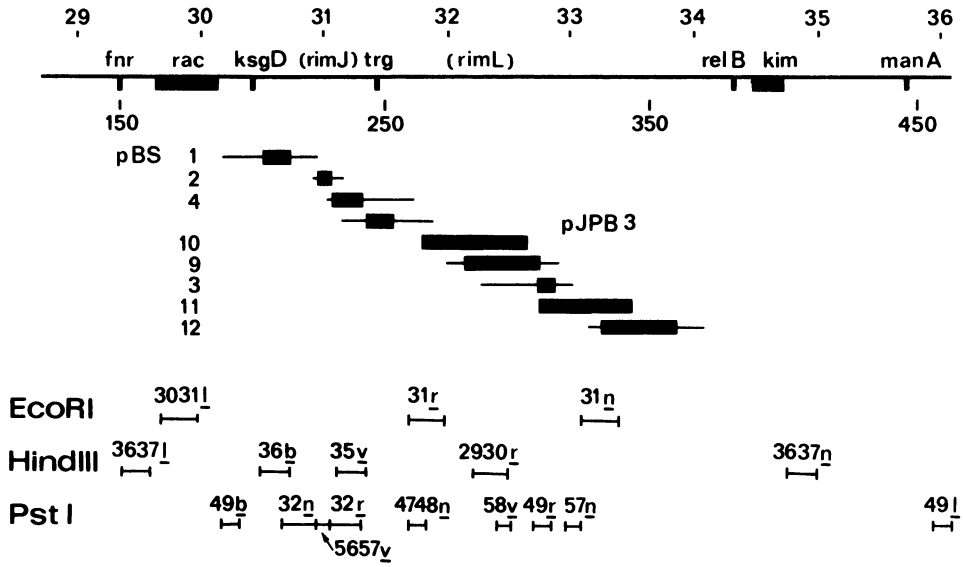

FIG. 1. Genetic map of the terminus region and location of the cloned fragments. The genetic scale, in minutes, and the physical scale, in kb, are shown above and below the map, respectively. These scales and the positions of genes *fnr*, *trg*, and *manA* and of the defective prophages *rac* and *kim* (boxed areas) are from Bouché et al. (6). The locations of *rimJ* and *relB* are as placed on the map of Bachmann and Low (1). Data on *ksgD* and *rimL* are taken from Fouts and Barbour (8) and Isono and Isono (12), respectively. Plasmid inserts (large bands) and regions further checked by Southern blot hybridization (thin bands) are shown to the same scale. The data for pJPB6 are from Bouché et al. (6). The names and positions of the *Eco*RI, *Hin*dIII, and *Pst*I fragments mentioned in the text are taken from Bouché (5) and are shown in the lower part of the figure.

were either screened for sensitivity to tetracycline (Tet^s) and replicated on gridded nitrocellulose filters (diameter, 85 mm) or directly established by filtration. Handling of master filters and replicas was as described by Hanahan and Meselson (10). For colony hybridization, a replica filter with approximately 1-mm-diameter colonies was transferred onto an L-broth agar plate containing 200 μg of chloramphenicol (Cam) per ml and incubated at 37°C for 20 h to amplify plasmid DNA. In situ colony lysis and DNA denaturation and neutralization were as described by Thayer (18). Cell debris was then washed off the filter with a stream of 10× SET (SET is 50 mM Tris [pH 8], 150 mM NaCl, 1 mM EDTA), and the filter was dipped for a few minutes at room temperature in a solution containing 10× SET, 10× Denhardt additives (7), and 100 μg of denatured calf thymus DNA per ml. The filter was then washed with 4× SET and dried at 80°C under vacuum. Hybridization (without prehybridization) was carried out essentially following Hanahan and Meselson (10).

Nomenclature and relation between physical and genetic distances. Names given to the fragments discussed below are from Bouché (5, Fig. 6). Positions are taken from this figure and expressed in kilobase pairs (kb). According to Bouché et al. (6), they can be converted into genetic map minutes by use of the equation, minutes = 2.15×10^{-2} position + 26.1 for positions > 66 kb (position of the *trp*::Mu insertion). Nick translation with ^3H-labeled precursors and hybridization to Southern blots were carried out as previously described (6).

RESULTS

Isolation of plasmid pBS1. *Hin*dIII-digested chromosomal DNA was fractionated by agarose gel electrophoresis, and the material which contained fragments H35*v*, H36*r*, H36*b*, H3637*l*, and H3637*n* from the terminus region (average size, 10.6 kb) (5) was eluted and ligated to plasmid pBR325 DNA linearized by *Hin*dIII and dephosphorylated. Upon transformation, the fraction of Tet^s colonies among Cam^r colonies was 84%. The plasmids from 40 colonies were analyzed by restriction endonucleases, and the patterns obtained were compared with those predicted from the map of the terminus region. One plasmid, pBS1, yielded a digestion pattern consistent with the insertion of fragment H36*b* (position, 204 to 214 kb). The restriction map of this plasmid is shown in Fig. 2. Hybridization of ^3H-labeled pBS1 to *Eco*RI$^-$, *Hin*dIII$^-$, or *Pst*I-digested DNA of strain CB0129 gave the results shown in Table 1. They agree with the predictions of the chromosomal map between positions 197 and 225 kb.

Isolation of plasmid pBS2. A mixture of *Pst*I chromosomal fragments, which include P5657*v*, P57*n*, and P58*v* (average size, 5.5 kb), was ligated to *Pst*I-treated, dephosphorylated pBR325 DNA. A total of 78% of the Cam^r

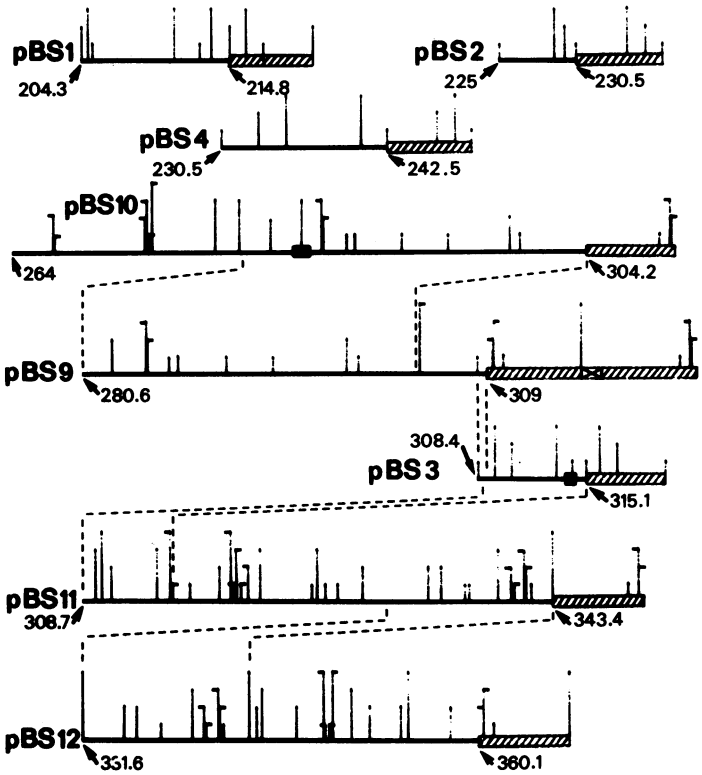

FIG. 2. Physical maps of the hybrid plasmids. Assignment of DNA: terminus region DNA, thin lines; insertion sequences, thick lines; vector DNA, hatched lines; unassigned DNA, crossed lines. The orientation of insertion sequences is arbitrary. *Bam*HI (for pBS9, pBS10, pBS11, and pBS12 only), *Eco*RI, *Hin*dIII, and *Pst*I sites are indicated by vertical lines of decreasing length. Dashed lines show the overlapping of the inserts. The physical positions of the inserts on the chromosomal map, in kb, are indicated at their ends.

transformants were Amp^s. The plasmids from 28 colonies were analyzed as above. One, named pBS2, had a size and digestion pattern consistent with the presence of fragment P5657*v* (Fig. 2). Results of the hybridization experiment are shown in Table 1. They confirm both the identity of the insert and the chromosomal map between positions 223 and 235 kb.

Isolation of plasmid pBS3. Chromosomal fragments including P47*r*, P4748*n*, P49*b*, P49*l*, and P49*v* (average size, 7.3 kb) were ligated with pBR325, and transformants of C600 were screened as for pBS2. A total of 73% of the Cam^r colonies carried hybrid plasmids. Twenty-eight of these were analyzed by digestion with *Pst*I + *Eco*RI or *Pst*I + *Hin*dIII. One plasmid appeared to contain fragment P49*v*. However, after large-scale preparation of this plasmid, pBS3, an additional *Pst*I fragment of 0.7 kb was detected. A map of the plasmid is shown in Fig. 2. When labeled pBS3 DNA was hybridized to digested chromosomal DNA of CB0129, fragments with the sizes and relative intensities expected from the map of the terminus between positions 286 and 320 kb were observed. In addition, 8 bands were visible in the *Eco*RI as well as in the *Hin*dIII lanes, and 15 bands were visible in the *Pst*I lane (Fig. 3, pBS3 lanes 1 to 3). Their sizes are listed in Table 1. Thus, a sequence of about 0.7 kb carrying a *Pst*I site and present at about eight copies per chromosome was inserted into pBS3. It should be noted that the pattern shown in Fig. 3 clearly resembles the pattern of chromosomal IS*1*-carrying fragments of *E. coli* K-12 strain JE5519 (16), suggesting that the additional fragment is an insertion of the transposable element IS*1*.

Isolation of plasmid pBS4. Chromosomal fragments that include P32*n* and P32*r* (average size, 12 kb) were ligated to pBR325 and transformed into C600. The yield of Tet^r Amp^s hybrid plasmids was 25%. Plasmid DNA from 28 colonies was run on an agarose gel, transferred to a nitrocellulose filter, and hybridized to chromosomal DNA labeled at the end of a synchronized replication cycle (5). Four plasmids yielded a strong response and two yielded a weak positive response. Upon restriction endonuclease analy-

TABLE 1. Sizes of the chromosomal DNA fragments hybridizing to plasmids pBS1, pBS2, pBS3, and pBS4

Probe	CB0129 DNA digestion	Expected sizes (kb)[a]	Observed sizes (Kb)[a]
pBS1	*Eco*RI	6.1, 2.7, 7.15, 15.8	6.1, 2.6, 7, 15.5
	*Hin*dIII	10.55	10.5
	*Pst*I	7.65, 12.35, 9.15	7.8, 12, 8.8
pBS2	*Eco*RI	5.1, 6.25	5, 6.4
	*Hin*dIII	6.95, 3.5	6.9, 3.55
	*Pst*I	5.5	5.5
pBS3	*Eco*RI	4.55, 6.1, 24.4	4.6, 6.1, 25+28, 21, 16, 14.5, 6.4, 5, 4, 3.75
	*Hin*dIII	8.1, 12.05	8.2, 12+26, 21, 17.5, 11.5, 11, 5.2, 4.1, 1.5
	*Pst*I	6.9	7.2+17.5, 14.5, 13.7, 12, 10, 6.8, 6.4, 5.8, 4.5, 3.7, 3.3, 1.7, 1.55, 1, 0.85
pBS4	*Eco*RI	5.4, 6.25, 20.3	5.3, 6.4, 21
	*Hin*dIII	11.25, 3.5	11.2, 3.5
	*Pst*I	12	12

[a] Expected fragments, taken from the map of the terminus region, are listed by order of decreasing homology with the plasmid, and outer fragments are underlined. Observed fragments are listed by order of decreasing apparent intensity of hybridization. Sizes of the extra bands hybridizing to pBS3 are given by order of size after a plus sign.

sis, two of the strongly responding plasmids proved to contain fragment P32*r* in opposite orientations. The map of one of these plasmids, pBS4, was constructed (Fig. 2). Hybridization of pBS4 to digested chromosomal DNA yielded the fragment sizes and relative intensities expected for the region between positions 229 and 261 kb (Table 1).

Isolation of plasmid pBS9. Three hundred Amp^r Tet^s colonies were isolated after transduction from a pool of encapsidated hybrid cosmids and established on a gridded nitrocellulose filter (85-mm diameter). A replica filter was hybridized, as indicated in Materials and Methods, to a pool of *Hin*dIII fragments of about 13.5 kb coming from DNA labeled at the end of a synchronized replication cycle. This pool contained a total of 88,000 cpm largely specific for fragment H2930*r* (position, 285 to 298 kb). After a 2-week exposure of the film, two colony prints showed a positive signal. Corresponding colonies from the master filter contained the same plasmid, pBS9, whose restriction map is shown in Fig. 2. Plasmid pBS9 carries the chromosomal sequences from positions 281 to 309 kb inserted between inverted repeats of the vector. Approximately 1.5 kb of DNA of unknown origin separates the vector moieties on the opposite side of this repeat. When hybridized to chromosomal DNA, pBS9 revealed all bands expected from the chromosomal map between positions 273 and 315 kb. In addition, 11, 8, and 12 other bands were visible in the *Eco*RI, *Hin*dIII, and *Pst*I lanes (Fig. 3, pBS9 lanes 1 to 3) and accounted for 40% of the total bound radioactivity, or approximately 20 kb of homologous DNA. It is reasonable to assume that one or a few of these extra bands are homologous to the 1.5 kb separating the vectors, but this is not sufficient to account for the number and overall intensity of the supernumerary bands. The nature of the repeated DNA involved has not been determined.

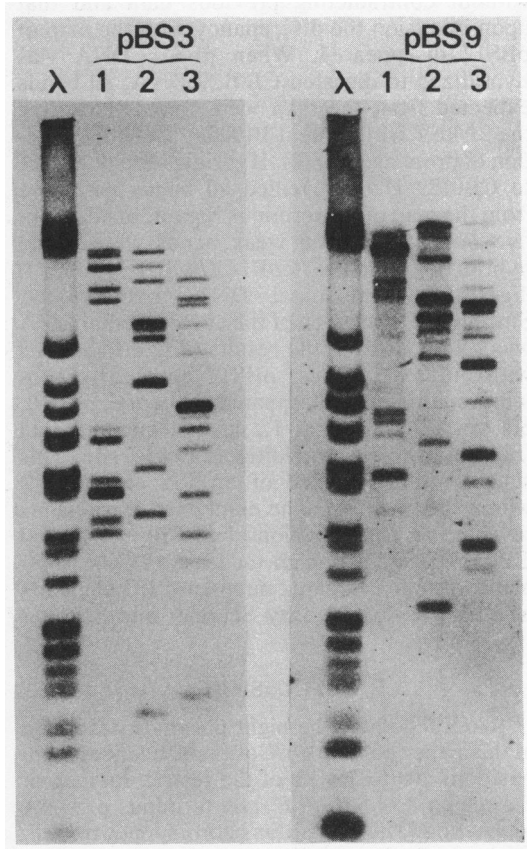

FIG. 3. Southern blot hybridization of CB0129 DNA to pBS3 (left) and pBS9 (right). Lane λ, Mixture of ^{3}H-labeled phage λ *c*I857 *S7* DNA digested by *Eco*RI, *Hin*dIII, or *Hin*cII. Lanes 1, 2, and 3, *Eco*RI-, *Hin*dIII-, or *Pst*I-digested CB0129 DNA, respectively. Sizes of the bands are listed in Table 1.

Isolation of plasmids pBS10, pBS11, and pBS12. Approximately 500 Amp^r colonies obtained after transduction from the pool of hybrid cosmids were established on a nitrocellulose filter by filtration. A replica filter was hybridized to a pool of in vivo labeled *Eco*RI fragments with sizes centered around 14 kb containing fragments E31*r*, E31*n*, and E3031*l* (total input, 240,000 cpm). Thirteen colonies giving a positive signal were obtained and analyzed. Three plasmids, pBS10 (one clone), pBS11 (one clone), and pBS12 (three clones), were thus obtained. Maps of these plasmids, including the position of the *Bam*HI sites, are shown in Fig. 2.

The inserts carried by these plasmids and by pBS3 and pBS9 overlap and, taken together, represent a DNA segment of 97 kb. Restriction endonuclease mapping of pBS12 revealed a discrepancy with the chromosomal map of the region of the terminus. We then noted that the 9.3-kb fragment containing P45*b* and the adjacent 1.75-kb *Pst*I fragment could be inverted without contradicting previous data and that upon inversion the discrepancy with the map of pBS12 disappeared. When pBS12 DNA was hybridized to digested CB0129 DNA, all bands expected from the map were found, including the 21-kb *Pst*I fragment P1920*l* toward the position of prophage *kim* (6). Hybridization of pBS11 to CB0129 DNA revealed all bands predicted from the map of the terminus region. In addition, five, four, and seven weak bands were found hybridizing from the *Eco*RI-, *Hin*dIII-, and *Pst*I-digested chromosomal DNA, respectively. Since partial digestion of the chromosomal DNA was ruled out from the results of hybridizations with different probes, pBS11 apparently contains an unidentified repeated sequence. pBS10, but not pBS9, carries 1.2 kb of extra material (Fig. 2), bringing an additional *Eco*RI site. This suggests the presence of an IS*5* element (4) within the fragment. The number of extra bands revealed by hybridization of pBS10 to digested CB0129 DNA was high (at least 12), in agreement with the estimated number of IS*5* elements in *E. coli* K-12 given by Schoner and Schoner (17).

DISCUSSION

Taken together, the eight plasmids described in this paper contain 86% of the sequences from positions 204 to 361 kb of the restriction map of the region around the *E. coli* terminus of DNA replication. These positions correspond, respectively, to 30.5 and 34 min on the genetic map. Figure 1 summarizes the positions of the inserted fragments and includes the position of pJPB3 (6). The present cloning leaves three gaps: 10.2 kb from the 214.8-kb end of pBS1 to the 225-kb end of pBS2, 0.8 kb from the 242.5-kb end of pBS4 to the 243.3-kb end of pJPB3, and 11 kb from the 253-kb end of pJPB3 to the 264-kb end of plasmid pBS10. Together with the additional Southern blot hybridizations, our results indicate that, except for a map inversion between positions 328 and 337 kb, the restriction map of this part of the chromosome previously published (5) is correct.

The point of termination of replication in strain LN681 has been approximately located in the region of the chromosome carried by plasmid pBS4, with a lower probability of being within pBS2 or pJPB3 (6). ColE1-related plasmids, as these three plasmids are, replicate unidirectionally, even if they carry the weak terminus of plasmid R6K (2). Thus, if a termination site exists in the 31- to 31.4-min region, it is either weakly functional or not functional when cloned or has been inactivated during the construction.

Whereas the cloning of fragments in the region of the terminus provides little information on the process of replication termination, plasmids carrying various segments of the region of the terminus and of the "silent" region between 31 and 34 min should be a useful tool for the study of gene expression and DNA rearrangements. In particular, we have used plasmids pBS1, pBS2, and pBS4 to elucidate the structure of an F′ factor derived from Hfr B7. This analysis is reported in the accompanying paper (20).

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