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The terminus region of the *Escherichia coli* chromosome contains two separate loci that exhibit polar inhibition of replication

(P2 *sig5* prophage/plasmids/DNA·DNA hybridization/DNA synthesis)

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ABSTRACT **The terminus region of the chromosome of *Escherichia coli* contains two separate sites, called *T1* and *T2*, that inhibit replication forks. *T1* is located near 28.5 min, which is adjacent to *trp*, and *T2* is located at 34.5–35.7 min on the opposite side of the terminus region, near *manA*. The sites act in a polar fashion, and replication forks traveling in a clockwise direction with respect to the genetic map are not inhibited as they pass through *T1* but are inhibited at *T2*. Similarly, counterclockwise forks are not inhibited at *T2* but are inhibited at *T1*. Consequently, forks are not inhibited until they have passed through the terminus region and are about to leave it. Studies with deletion strains have located *T2* within a 58-kilobase interval, which corresponds to kilobase coordinates 387–445 on the physical map of the terminus region.**

The terminus region of the chromosome of *Escherichia coli* is located directly opposite the origin of replication on the circular genetic map (1). One of the most interesting features of this region is that it inhibits replication forks that are traveling in either a clockwise or counterclockwise direction with respect to the map (2–5). When origins of replication located near the terminus region were used (2–5), inhibition of replication forks occurred somewhere in the interval between *trp* (28 min) and *manA* (36 min). Although the function of this inhibition is unknown, its presence suggests that the terminus might encode partitioning, decatenation, or cell-division control sites, and impediment of replication forks in this region evolved to ensure efficient use of such sites (6).

More recent experiments using replication cycles initiated at *oriC* (7) indicated that the last DNA to be replicated, and consequently the region where forks meet most frequently, was located near 31.2 min. Based on these results, it generally has been assumed that the replication block (*terC*) is located at this position (1). As discussed by Bouché *et al.* (7), however, other interpretations of the data can be made. Therefore, identification of the region of most frequent fork encounter does not necessarily identify the site of replication-fork inhibition.

We report here, as do de Massy *et al.* in an accompanying report in this issue (8), that replication forks are not inhibited at a single site in the middle of the terminus region. Instead, there are two inhibition sites, *T1* and *T2*, which are located at the outer edges of the terminus region. These sites are polar, and they only inhibit replication forks that have passed through the terminus region and are about to leave it. Specifically, clockwise traveling replication forks are inhibited at *T2* at 34.5–35.8 min, near *manA*, and counterclockwise traveling forks are inhibited at *T1* at 28.5 min, near *trp*.

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MATERIALS AND METHODS

Bacterial Strains. All replication fork assays were performed with strains PK998 or PK1012 and derivatives containing the indicated deletions. These temperature-sensitive strains are *dnaA* mutants and contained temperature-sensitive P2 *D4 c5 sig5* bacteriophage, which we call P2 *sig5* prophage, integrated near *galK* (16 min) or *metG* (47 min), respectively. They were identical to PK583 and PK504, which have been described (2, 3), except that the *rac* cryptic prophage has been removed (9).

Genetic Procedures. Fig. 1 shows the locations of the deletions described in this paper. The end points were determined by Southern hybridizations with appropriate probes (11). PK1441, containing deletion 1441, was isolated from a strain that contained phage λ reverse *c*I857 [inserted at kilobase (kb) 164] and *zdc-235*::Tn*10* (at kilobase 290). Derivatives were selected that were temperature resistant and tetracycline sensitive (12). PK1463 was isolated from a strain containing *zdd-230*::Tn*9* and *zdc-235*::Tn*10* (13), and PK1738 was isolated from a strain containing *zdd-230*::Tn*9* and *zde-234*::Tn*10*. In both cases, tetracycline-sensitive derivatives were isolated and screened further. PK2035 and PK2038 were derived from a *ptsG* strain that contained *zdc-235*::Tn*10* (at kb 290) and *zdf-237*::Tn*10* (at kb 445). Presence of *ptsG* allowed selection for *dgsA* (at kb 425) mutants, which grew anaerobically on glucose as the carbon source (14).

Bacteriophage P1 was used to transduce deletions into PK998 and PK1012. The presence of the deletion in recipient strains was verified by both genetic and Southern blot analysis.

DNA·DNA Hybridization Assay. Cells were grown at 28°C in M9 medium supplemented with required amino acids (80 μg/ml) and thymine (2 μg/ml). At A_{450} of ≈0.45, cells were collected on filters (HAWP, Millipore), washed once with M9 minimal medium, and resuspended at the same concentration in medium lacking all required amino acids except arginine. Incubation was continued for 170 min at 28°C to allow preexisting forks to complete replication cycles.The culture was then shifted to 42°C for 10 min to inactivate the *dnaA* product and induce initiation from the P2 *sig5* prophage. Required amino acids and [^{3}H]thymine were then added (final specific activity, 8.1 Ci/mmol). Samples (2 ml) were removed at indicated times, washed four times with an equal volume of TE buffer (25 mM Tris·HCl/10 mM EDTA, pH 8.0), and resuspended in 1.0 ml of TE buffer containing lysozyme (5 mg/ml). After incubation for 5 min at room temperature, Pronase and $NaDodSO_4$ were added to a final concentration of 100 μg/ml and 1%, respectively, and the samples were incubated at room temperature overnight.

Samples were prepared for hybridization in the following manner. Four hundred microliters (1–2 × 10^7 cpm) of cellular [^{3}H]DNA was hydrolyzed with 250 mM HCl in a final volume of 1.0 ml; 200 μl of 2.5 M NaOH was added to neutralize the

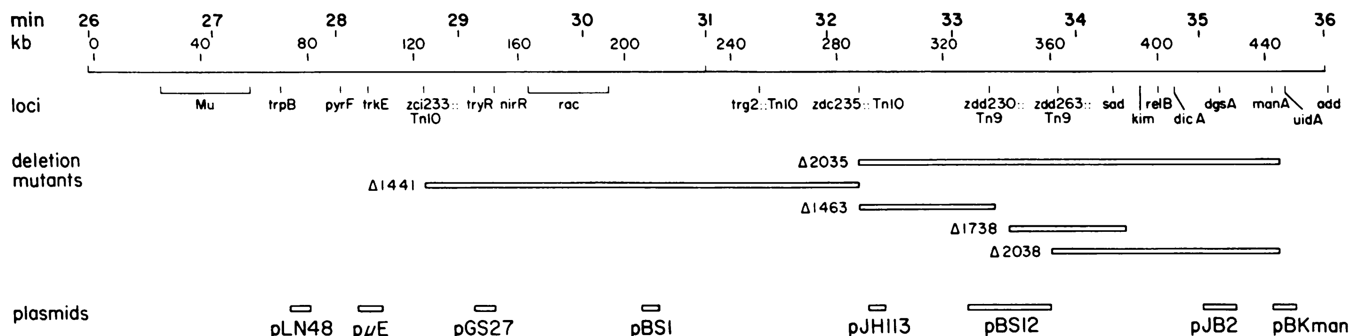

FIG. 1. Map of the terminus region of the *E. coli* chromosome. Physical coordinates (kb) are from Bouché (10), and genetic coordinates (min) are from Bachmann (1). Large open lines represent deletions described in this report. Small open lines represent plasmid clones of fragments from the terminus region that were used in DNA·DNA hybridizations.

acid and denature the DNA. At this time 2 × 10^7 cpm of ^{32}P-nick-translated chromosomal DNA (obtained from a stationary culture of the same strain) was added to the sample. After 5 min at room temperature, 100 μl of 2.5 M HCl was added to neutralize the base, and 200 μl of 2 M sodium phosphate buffer (pH 6.8) was added to stabilize the pH. The sample was then brought to 4× NaCl/Cit (1× = 0.15 M NaCl/0.015 M sodium citrate, pH 7), 4× Denhardt's solution (11), 50% formamide, and 1% $NaDodSO_4$ in a final volume of 8.0 ml.

Plasmid probes were prepared by the alkaline hydrolysis method essentially as described by Maniatis *et al.* (11). A list of the plasmid probes used in these experiments is presented in Table 1. Plasmids were banded in CsCl gradients at least twice to remove contaminating chromosomal DNA. Purified probes were loaded on nitrocellulose by using a slot-blot apparatus (Schleicher & Schuell) by the following procedure. Plasmid, 0.6 pmol in 200 μl, was hydrolyzed for 5 min in 0.25 M HCl. The acid was neutralized, and the fragmented plasmid was denatured by the addition of 0.4 M NaOH to a final volume of 500 μl. Tris/salt (1.0 ml; 0.75 M Tris·HCl/1.5 M NaCl, pH 7.5) was added, and 500 μl of DNA was loaded per slot (three slots per plasmid probe; 0.2 pmol of plasmid per slot). Each slot was then washed with 500 μl of Tris/salt and, after drying the blots, they were baked for 2 hr at 80°C

Table 1. Plasmids used in DNA·DNA hybridization assay

Plasmid number	Position, min	Plasmid designation	Reference or source
1	1.8	pJE22606	15
2	8.0	pSKS105	16
3	23.8	pLC36-1	17
4	27.7	pLN48	18
5	28.3	pμE	W. Epstein
6	29.1	pGS27	19
7	30.5	pBS1	20
8	32.6	pJH113	21
9	33.5	pBS12	20
10	35.2	pJB2	22
11	35.8	pBKman	21
12	37.5	pB1	23
13	41.0	pLC1-28	17
14	48.6	pLC3-46	17
15	53.9	pSE301	24
16	63.0	pLC2-5	17
17	69.0	pHA11	25
18	76.0	pLC31-16	17
19	84.6	pLC26-3	17
20	85	pLC21-35	17
21	92.0	pDR2000	26

P2 *lg cc vir22* (27) was used as the probe for determining replication at the P2 *sig5* prophage origins located at either 16 or 47 min.

and prehybridized overnight at 42°C in 50% formamide containing 5× NaCl/Cit, 5× Denhardt's solution, 50 mM sodium phosphate buffer (pH 6.8), and 1% $NaDodSO_4$. After prehybridization, the solution was replaced with the hybridization mixture containing the labeled DNAs and allowed to hybridize for 36 hr at 42°C. Blots were washed four times in 2× NaCl/Cit containing 0.1% $NaDodSO_4$ for 5 min per wash at room temperature and twice in 0.2× NaCl/Cit containing 0.1% $NaDodSO_4$ at 55°C for 45 min each wash. Slots were cut out and assayed in a scintillation cocktail (Omnifluor; New England Nuclear) to determine the ^{3}H/^{32}P ratios for each plasmid.

Triplicate samples of plasmid pBR322 were included on each blot to serve as a control for background hybridization of both ^{3}H and ^{32}P. The average number of cpm bound to the pBR322 control was subtracted from the experimental values prior to calculating the ^{3}H/^{32}P ratio for each slot. The mean and standard error were then determined from the three corrected values obtained for each plasmid probe.

RESULTS

In all experiments, the P2 *sig5* prophage was used to initiate replication forks from positions close to the terminus region. P2 *sig5* prophage inserted at 16 min (near *gal*) was used to study clockwise-traveling replication forks in the terminus region, and an insertion at 47 min (near *metG*) was used to study counterclockwise-traveling forks. As described in previous publications (2, 3, 21), initiation at the P2 *sig5* prophage was induced in conditions that prevented initiation of replication forks from *oriC*. The asymmetric placement of the P2 *sig5* prophage with respect to the terminus region was advantageous because replication forks originating from the prophage could be monitored as they passed through the terminus without interference from forks approaching from the other side.

We have used a greatly expanded repertoire of plasmids in our assay to determine the amount of replication in the terminus and at other positions around the chromosome (details of these plasmids are given in Table 1). Consequently, we have increased the sensitivity of the marker-frequency analysis considerably. Additionally, all strains used in the experiments reported here lacked the *rac* prophage, which is located at 30 min (1). This avoided complications due to the potential induction of this prophage, which could lead to its excision (9) or to replication initiated at *oriJ* (28).

Fig. 2 shows the results of an experiment in which replication was initiated from the P2 *sig5* prophage located near the *gal* locus. Samples were taken at 90, 135, and 180 min after induction of the prophage replication origin. The clockwise-traveling replication forks proceeded normally through most of the terminus region but were severely inhibited in the

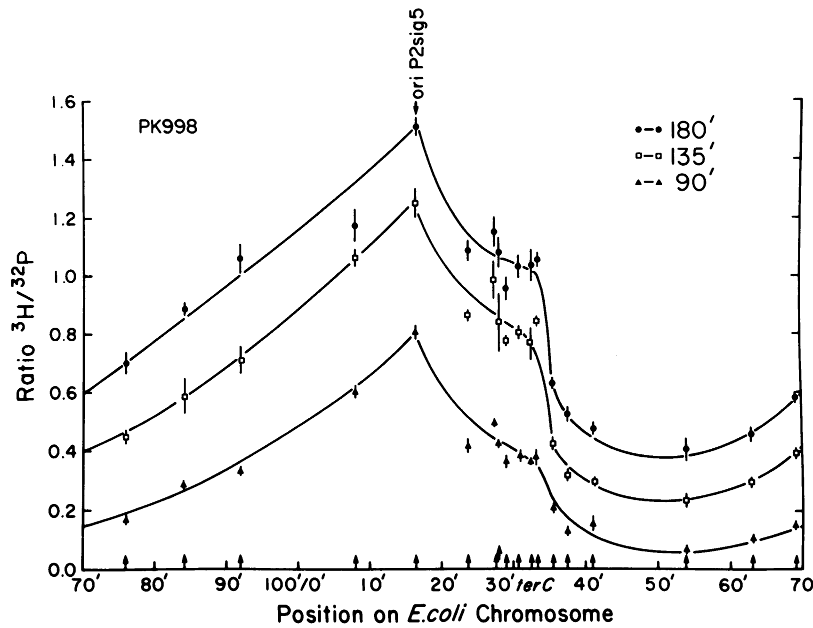

FIG. 2. Termination of replication in strain PK998 (P2 *sig5* prophage is located at 16 min). The points represent the amount of DNA replication at 90, 135, and 180 min after induction of the prophage. The plasmids used were 2–9, 11–13, 15–19, and 21 in Table 1.

interval between 33.5 min (plasmid pBS12) and 35.8 min (plasmid pBKman). This region corresponds to kb 350–445 on the Bouché map (10). Accumulation of replication forks at the replication block did not perceptibly alter the location at which subsequent replication forks were halted. As has been previously observed, however, replication forks slowly proceeded through the inhibition site (2, 29), and the clockwise traveling forks eventually met the counterclockwise forks in the region near 55 min.

A comparable experiment was done to determine the location of the region in which counterclockwise-traveling replication forks were inhibited (Fig. 3). In this experiment, the P2 *sig5* prophage was inserted near *metG*. Counterclockwise-traveling forks traversed most of the terminus region but were severely inhibited in the region between 29.1 min (plasmid pGS27) and 27.7 min (plasmid pLN48). This region corresponds to kb 70–140 on the Bouché map (10). As observed with the clockwise block, the inhibition site was not altered by arrival of subsequent replication forks, and forks did proceed through the block. The counterclockwise-traveling forks met the clockwise-traveling forks in the region near 10 min.

Comparison of Figs. 2 and 3 shows that the site that blocks counterclockwise-traveling forks (*T1*) has no effect on clockwise-traveling forks; similarly, the site that blocks clockwise-traveling forks (*T2*) has no effect on counterclockwise-traveling forks. This indicates that the inhibition sites act in a polar fashion.

In order to characterize the inhibition sites further, we isolated and tested a number of deletions in the terminus region. Our goal in these studies was to obtain deletions that define *T1* and *T2* to regions of 15 kb or less, at which stage it should be possible to study the sites further in plasmids. Such a deletion approach is feasible, since the terminus

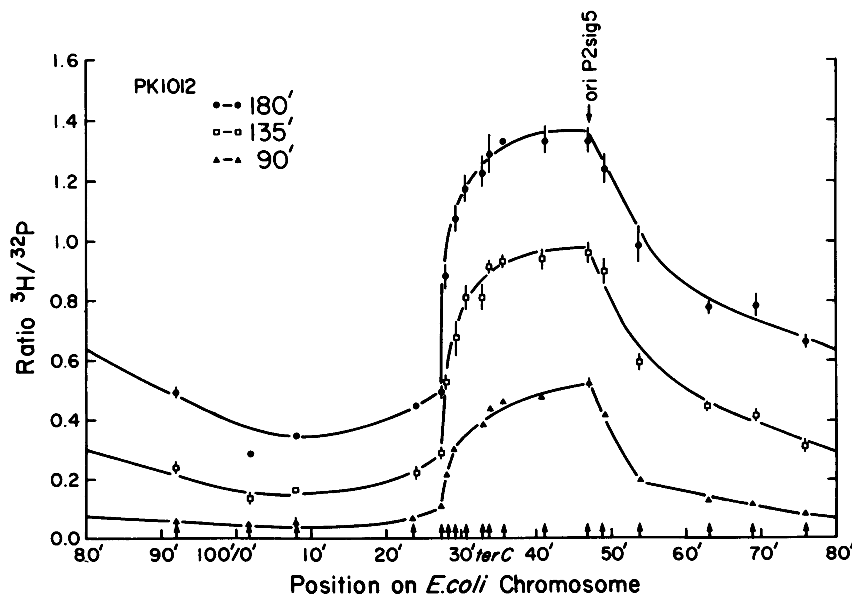

FIG. 3. Termination of replication in strain PK1012 (P2 *sig5* prophage is located at 47 min). The points represent the amount of DNA replication at 90, 135, and 180 min after induction of the prophage. The plasmids used were 1–9, 11, 13–18, and 21 in Table 1.

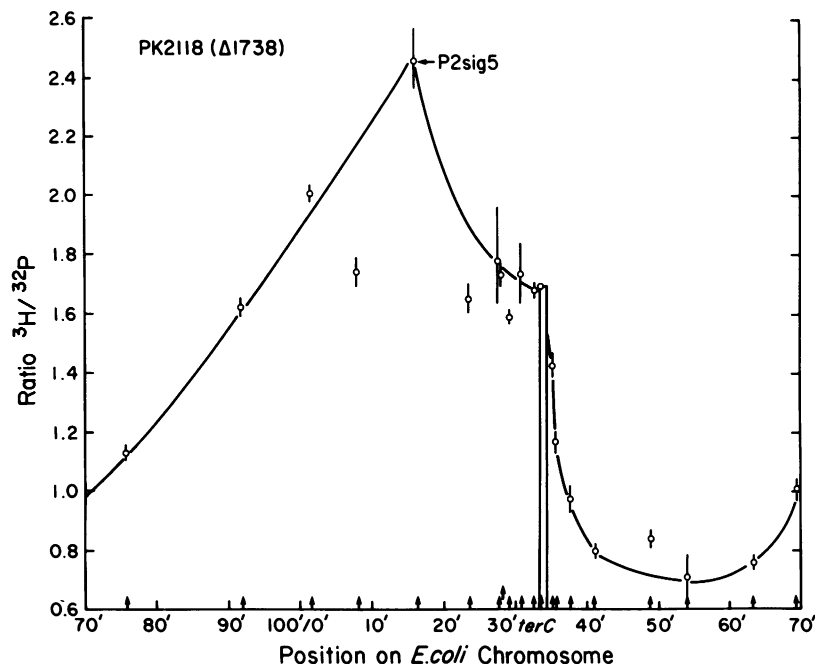

FIG. 4. Termination of replication in strain PK2118, which contains deletion *1738* (kb 350–390) and P2 *sig5* prophage located at 16 min. The boundaries of the deletion are indicated by the vertical lines. The points represent the amount of DNA replication 180 min after induction of the prophage. The plasmids used were 1–18 and 21 in Table 1.

region is not essential, and it is possible to isolate deletions in which neither inhibition site functions (21).

By use of various deletions (1463, 1738, 2035 and 2038; see Fig. 1) we can presently limit *T2* to a region of <60 kb. Although deletion 2035 (at kb 290–445) did not provide a more specific localization than the experiments described above, it set the limits for further analyses, since loss of that interval also removed *T2* (data not shown). Deletions 1463 (at kb 290–342) and 1738 (at kb 348–387) subdivided the region corresponding to the left-hand part of deletion 2035. One (1738) removed the interval in which inhibition first occurred, and the other (1463) deleted the region immediately upstream. Neither of these deletions affected *T2*, however, and Fig. 4 shows the results obtained with a strain containing deletion 1738. *T2* was no longer functional in strains harboring deletion 2038 (at kb 360–445), which removed the region corresponding to the right-hand side of 2035 (Fig. 5). Combining the results obtained with deletions 2038 and 1738 places *T2* between kb 387 and 445.

Although we have isolated a number of deletions that remove parts of the left-hand side of the terminus region, we have not yet isolated small deletions that remove *T1*. The most informative deletion is 1441 (at kb 123–290), in which *T1* still functioned (data not shown). de Massy *et al.* (8) have

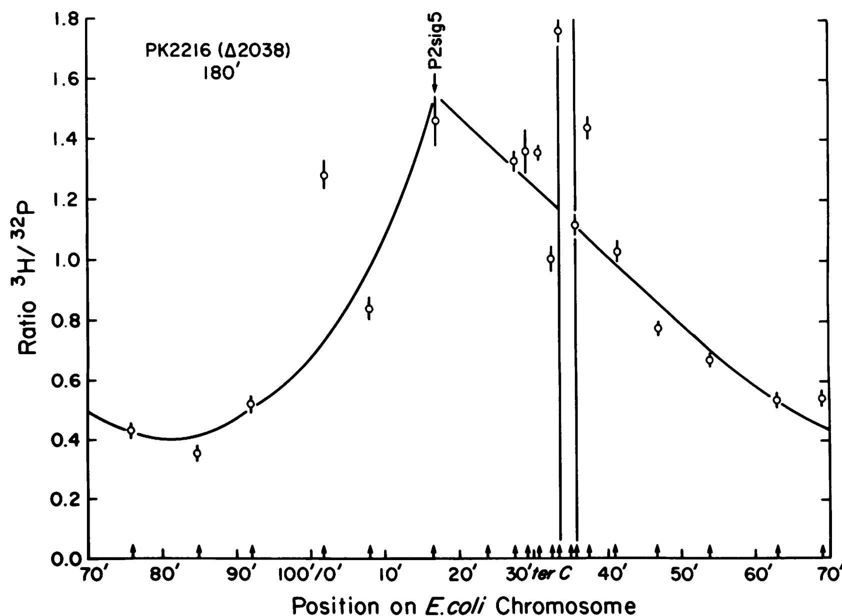

FIG. 5. Termination of replication in strain 2216, which contains deletion *2038* (kb 360–445) and P2 *sig5* prophage located at 16 min. The boundaries of the deletion are indicated by the vertical lines. The points represent the amount of replication 180 min after induction of the prophage. The plasmids used were 1, 2, 4, 6–18, 20, and 21 in Table 1.

isolated comparable deletions that removed kb 119–162 and 133–246, and *T1* still functioned in strains harboring those deletions. This shows that *T1* is to the left of kb 123.

DISCUSSION

We demonstrate here that clockwise- and counterclockwise-traveling replication forks are not inhibited in the same part of the terminus region of the *E. coli* chromosome. Clockwise-traveling forks are inhibited at *T2*, which is between 34.5 and 35.7 min on the side of the terminus region near *manA*. Counterclockwise-traveling forks are inhibited at *T1*, which is on the opposite side of the terminus region at 28.5 min near *Trp*. The two regions that inhibit replication forks are consequently separated by ≈6 or 7 min on the genetic map. The mechanism by which replication forks are inhibited is presently unknown, but it should be stressed that the blocks act in a polar fashion. Clockwise-traveling forks are not affected as they travel through *T1*, but they are inhibited by *T2*. Similarly, counterclockwise-traveling forks are not affected by *T2*, but they are inhibited by *T1*.

The location of the replication blocks indicates that normal replication cycles usually terminate with the collision of replication forks in the interval between *T1* and *T2*. The clockwise genetic distance from *oriC* to *T2* is ≈51 min, and the counterclockwise distance from *oriC* to *T1* is ≈56 min. If these distances are actually physically equivalent (*ca.* 53.5 min of DNA), then one fork would have to proceed at least 15% faster than the other (53.5 vs. 46.5 min of DNA replicated) for an inhibition site to be used. Autoradiographic studies have shown that the majority of chromosomes exhibit bidirectional replication rates that differ by <15% (refs. 30 and 31; unpublished data). If replication cycles are frequently terminated between *T1* and *T2* without a block site being used, the questions arise of how essential the sites are, how often they are used, and why they evolved. It should be added that, if the physical distances from *oriC* to *T1* and *T2* actually were 56 and 51 min, respectively, *T2* would be used more frequently than *T1*. This does not fit the data of Bouché *et al.* (7), who demonstrated that the average position of fork encounter was near 31.2 min, midway between *T1* and *T2*.

The arrangement of the replication blocks in *E. coli* is considerably different from that observed in other terminus regions that have been studied. In plasmid R6K, there is only one termination site, which inhibits replication forks traveling in either direction (32). A similar situation possibly occurs in *Bacillus subtilis* in which replication forks traveling counterclockwise (33–35) and clockwise (36) halt in the same restriction fragment, and only one block site has been identified. A second site might exist, however, since clockwise-traveling replication forks are the first to arrive at this site (33), and their presence would inhibit counterclockwise-traveling forks. Strains in which the counterclockwise-traveling forks are the first to arrive in this region have not yet been tested. Regardless, a comparison of the features of the terminus regions of *E. coli* and *B. subtilis* should provide insights into the important features of these regions.

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