

More Precise Mapping of the Replication Origin in *Escherichia coli* K-12

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The origin of replication in *Escherichia coli* K-12 was mapped by determining the rate of marker replication during a synchronous round of replication. Four isogenic strains were made lysogenic for λind^- and for phage Mu-1, with Mu-1 integrated into a different chromosomal location in each strain. Cultures were starved for amino acids to allow completion of chromosome replication cycles and then starved for thymine in the presence of amino acids, and a synchronous cycle of replication was initiated by the addition of thymine. Samples were exposed to radioactive thymidine at intervals, deoxyribonucleic acid was extracted, and the rate of marker replication was determined by deoxyribonucleic acid-deoxyribonucleic acid hybridization to filters containing Mu-1, λ , and *E. coli* deoxyribonucleic acid. The results confirm that the origin of replication is near *ilv*. The travel times of the replication forks, calculated from the data obtained for cultures with doubling times of approximately 40 and 61 min, are 40 and 52 min, respectively.

The replication of the chromosome in an F⁻ strain of *Escherichia coli* is initiated at a fixed origin and proceeds bidirectionally. Several authors (3, 6, 7, 10, 13, 24) have located this origin near the marker *ilv*, which is at 75 min on the map (21). Others (9, 17, 19, 22, 23) have favored sites further away from that position. In the experiments presented here, we studied the replication of various markers during a synchronized cycle of replication to define more precisely the map position of the origin and to analyze the mode of displacement of the growing forks.

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MATERIALS AND METHODS

Bacteria and bacteriophage. The bacterial strains used in this study are listed in Table 1. The map positions of the markers used are shown in Fig. 1. Bacteriophages Mu-1, $\lambda cl^{857}S^7$, λind^- , and $\lambda imm21cI^-$ were from the collection of L. Caro. The growth of Mu-1 and $\lambda cl^{857}S^7$ stocks for preparation of deoxyribonucleic acid (DNA) has been described (3).

Growth media. Glucose M9 medium was used (1). Minimal medium was M9 supplemented with (per milliliter): thiamine, 0.5 μ g; thymine, 10 μ g; leucine,

100 μ g; and any additional requirement, 100 μ g. Casamino Acids medium was minimal medium containing 0.2% vitamin-free Casamino Acids (Difco). The growth rates of the strains in these media are shown in Table 1.

Medium changes were made by collecting cells on a Sartorius membrane filter, washing them with prewarmed M9 medium, and suspending them in the desired medium at 37 C.

Cell growth was monitored by adsorption at 450 nm in a Hitachi spectrophotometer or a Coulter counter, model B.

Pulse labeling. Cultures for pulse labeling were pipetted or poured rapidly into tubes containing the radioactive thymidine. To terminate a pulse the cultures were poured rapidly onto crushed, frozen medium containing 5 to 10% pyridine (12).

DNA extractions and DNA-DNA hybridization. DNA was extracted from bacteriophages Mu-1 and λ by freeze-thawing three times in 10^{-2} M ethylenediaminetetraacetic acid-0.1% sodium dodecyl sulfate. The DNA was purified by isopycnic centrifugation by the two-layer CsCl method (4).

Pulse-labeled DNA was extracted in 0.01 M tris(hydroxymethyl)aminomethane-0.05 M ethylenediaminetetraacetic acid (pH 7.6). Lysozyme (10 to 100 μ g) was added, the mixture was incubated for 10 min in ice, and Sarkosyl (Geigy) was added to 0.1% to lyse the cells. The lysate was inactivated with pancreatic ribonuclease (50 μ g/ml; Sigma Chemical Co.) for 30 min at 37 C, followed by Pronase (Calbiochem; grade B, 1 mg/ml) or proteinase K (Merck; 50 μ g/ml) for 1 to 2 h at 37 C. The lysate was incubated with additional ribonuclease (50 μ g/ml) for 30 min. Lysates were purified by centrifugation in CsCl. The purified DNA

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TABLE 1. Bacterial strains and their growth rates^a

Strain	Genotype	Site of Mu-1 integration	Doubling times (min)	
			Minimal	Casamino Acids
CB 0129	F- <i>leu thy thi</i>	None		
Mx 213	F- <i>leu thy thi ilv</i> (λind^-)	<i>ilv</i> (75 min)	54	38
Mx 222	F- <i>leu thy thi rha</i> (λind^-)	<i>rha</i> (77 min)	66	44
Mx 223	F- <i>leu thy thi thr</i> (λind^-)	<i>thr</i> (0,90 min)	65	39
Mx 239	F- <i>leu thy thi malA</i> (λind^-)	<i>malA</i> (66 min)	60	38

^a All strains were derived from strain CB 0129. The Mu-1 lysogens are derivatives of strains used in our previous study (3). These were cured of λ^+ by infection with $\lambda imm21 cI^-$. Colonies that had lost the λ immunity as a result of this infection were then lysogenized with λind^- (a noninducible strain of bacteriophage λ).

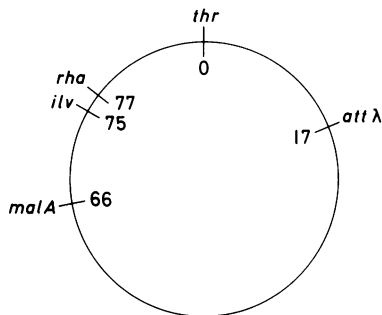


FIG. 1. Genetic map of *E. coli* K-12. The sites of prophage integration for the strains used in this study are shown (21).

was dialyzed against $0.1 \times SSC$ ($1 \times SSC = 0.15 M NaCl-0.015 M$ sodium citrate, pH 7) and stored at $-20 C$.

E. coli CB 0129 DNA was extracted by the method described above except that the lysates were deproteinized twice with phenol before purification in CsCl.

DNA concentrations were determined by absorption at 260 nm in a Zeiss spectrophotometer or by the diphenylamine reaction (5).

To prepare filters for hybridization, DNA was denatured in 0.15 N NaOH, neutralized with HCl, and loaded onto filters in $6 \times SSC$. Filters were dried, heated at $80 C$ under vacuum, and stored in a desiccator until use. Radioactive DNA was sheared with a Branson sonifier, denatured with NaOH, neutralized with HCl, made up to $4 \times SSC$, and diluted 1:1 with formamide (Merck). A 0.5-ml volume of the radioactive mixture, containing 5,000 to 10,000 counts per min, was added to each filter, which was then incubated for at least 2 days at $42 C$. Filters were washed first in $2 \times SSC-50\%$ formamide for 20 to 30 min, followed by two washes in $2 \times SSC$ (14). Filters were dried, and the radioactive DNA bound to the filters was determined with a toluene-Permablend III (Packard) or a Packard Tricarb liquid scintillation counter.

Samples of the labeled DNA were hybridized to filters supporting 1 or $2 \mu g$ of calf thymus, *E. coli*, λ , or Mu-1 DNA. After the nonspecific binding to calf thymus filters was subtracted, the counts binding to

E. coli, λ , and Mu-1 filters were summed, and the fraction of this total bound to Mu-1 and λ was determined. The data were calculated in this manner to reduce error caused by small differences in hybridization efficiency. The binding to *E. coli* DNA filters was 10 to 30% of the added radioactivity. To correct for nonspecific binding, pulse-labeled DNA from strain CB 0129 was used as a control in all experiments. The fraction of radioactivity hybridized to Mu-1 and λ filters was subtracted before the data were plotted.

Design of the experiment. Auxotrophs of *E. coli* deprived of required amino acids continue DNA synthesis until completion of the cycles of chromosome replication already initiated, but cannot initiate new cycles (15, 16). After restitution of the amino acids, new rounds of replication begin, at varying times depending on the age of each cell at the onset of amino acid deprivation, at the normal initiation site (2, 15, 16). In a thymine-requiring strain, this initiation can be synchronized by adding the required amino acids in the absence of thymine, thus allowing the synthesis of the proteins required for initiation while blocking DNA synthesis. Synchronous replication is initiated by addition of thymine (6, 23). This procedure is summarized in Fig. 2.

Using this technique, we synchronized chromosome replication in four *E. coli* strains lysogenized for both bacteriophages λ and Mu-1. The λ prophage was always present at the normal λ integration site, whereas the Mu-1 prophage was integrated at various sites (Fig. 1). During synchronous replication, samples of each culture were exposed to tritiated thymidine for a brief period. The DNA was extracted and hybridized to filters loaded with either λ or Mu-1 DNA, and the amount of radioactivity hybridizing to each was determined. Since DNA replication begins synchronously, the relative order of marker replication can be determined by the relative amount of radioactivity specific for each marker as a function of time after initiation.

Both prophages, Mu-1 (20) and λind^- (11), used in this study are noninducible. We determined that the number of either type of phage produced, after the period of thymine starvation, is less than 10^{-3} phage per bacterium. Therefore, phage induction cannot seriously influence our results.

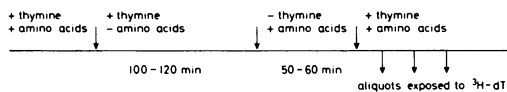


FIG. 2. Experimental procedure for synchronization of replication cycles.

RESULTS

Replication in glucose-minimal medium.

The cultures were grown in minimal medium and synchronized, and the replication pattern was determined (Fig. 2 and 3). The data in Fig. 3 are presented as the fraction of total radioactivity hybridized versus the time at which the radioactive pulse began.

Figure 3A shows the results for the strain with Mu-1 integrated into *ilv*. The Mu-1 prophage was replicated over a very short period beginning at initiation, the bulk of the Mu-1 having been replicated by min 4. The sharpness of *ilv* replication in Fig. 3A indicates good synchrony of initiation and the closeness of the prophage to the site of initiation. The background level of Mu-1 replication was probably a result of subsequent initiation events.

The prophage λ was not replicated at all until about 20 min. A maximal rate was attained at about 30 min and dropped to a minimum around 40 min. The absence of replication of λ before 20 min indicates that the chromosomes were aligned, since there were no "forgotten" replication forks, and that the DNA synthesis observed was not the result of repair replication. The prolonged period of replication of the prophage λ shows that the synchrony was rapidly lost (see below).

The prophage Mu-1 integrated into *rha* was replicated soon after initiation (Fig. 3B), but *rha* seems to be located further from the site of initiation than *ilv*. Maximal *rha* replication began soon after initiation, but it was clearly not confined to as brief a period as that of *ilv* and occurred later. At 4 min the rate of *rha* replication remained high, whereas the rate of *ilv* replication dropped to the background level. Thymine incorporation proceeded at the same rate in both the *ilv* and *rha* strains after synchronization (data not shown). This excludes the trivial possibility that the *rha* strain is delayed in initiation. A background level of replication of this marker was attained after about 8 min. Again the prophage λ was not replicated before 20 min.

The other markers, *malA* and *thr* (Fig. 3C and D), were replicated later; maximal rates were obtained at 8 to 10 min and 20 min, respectively. The fact that markers *rha*, *malA*, and *thr* were replicated soon after *ilv* indicates

that replication is bidirectional. In all cases, the prophage λ was replicated over a prolonged period after 20 min.

Replication in Casamino Acids medium.

The experiment was repeated with strains grown in Casamino Acids medium (see Table 1 for the growth rates in this medium). The results for cultures grown in this medium are shown in Fig. 4. *ilv* was replicated during a brief period immediately after initiation, followed by *rha*, *malA* and *thr*, in that order. The prophage λ was always replicated over a prolonged period after 15 min. Note that the late markers appear to have been replicated sooner in this medium than in minimal medium (see below).

DISCUSSION

Origin of replication. In our original report on bidirectional replication, we could not locate the origin more precisely than ± 5 min (3). From the data presented here, we can now assign a

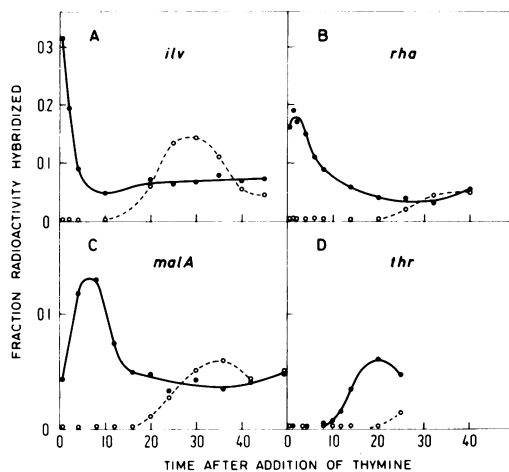


FIG. 3. Replication pattern in glucose-minimal medium. Strains Mx 213 (Mu-1 in *ilv*), Mx 223 (Mu-1 in *thr*), Mx 222 (Mu-1 in *rha*), and Mx 239 (Mu-1 in *malA*) were grown in glucose minimal medium, incubated in medium lacking amino acids for 2 h, and incubated in medium lacking thymine but containing amino acids for 50 min (the first three cultures) and 60 min (the last culture). Thymine, 4 $\mu\text{g/ml}$ for Mx 213 and Mx 223 and 10 $\mu\text{g/ml}$ for Mx 222 and Mx 239, was added to initiate replication. A 20-ml volume (each) of Mx 213 and Mx 223 was pulsed with 200 μl of ^3H -labeled thymidine (20 Ci/mmol) for 20 s, and 2.5 ml (each) of Mx 222 and Mx 239 was pulsed with the same amount of radioactivity for 60 and 120 s, respectively. The pulses were quenched, DNA was extracted, and the rate of marker replication was determined. The quantity of DNA added to each filter was approximately 1 μg for A and D and approximately 0.1 μg for B and C. (A) Mx 213, (B) Mx 222, (C) Mx 239, and (D) Mx 223 (Mu-1, ●; λ , ○).

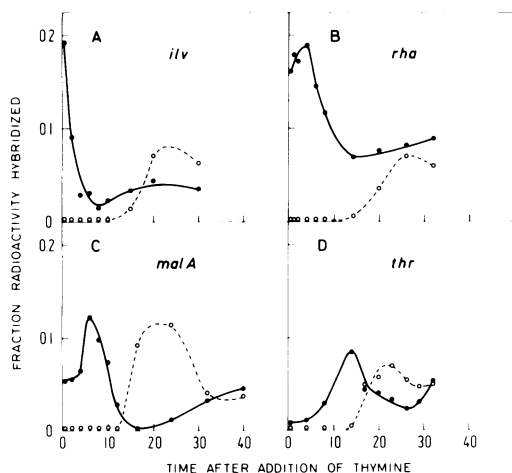


FIG. 4. Replication pattern in Casamino Acids medium. The four strains used in the experiment of Fig. 1 were grown in Casamino Acids medium deprived of amino acids for 100 min (120 min for Mx 239) and then deprived of thymine. After 50 min (60 min for Mx 239), thymine (10 $\mu\text{g}/\text{ml}$) was added to initiate replication. Samples of each synchronized culture (2.5 ml) were pulsed for 60 s with 200 μl of ^3H -labeled thymidine. The replication pattern was examined. Approximately 0.1 μg of DNA was added to each filter. (A) Mx 213, (B) Mx 222, (C) Mx 239, and (D) Mx 223 (Mu-1, ●; λ , ○).

more precise location. The first marker replicated is *ilv* followed very closely by *rha*. The *ilv* marker is replicated over a short period beginning at the time of addition of thymine. The exact timing of *rha* replication is difficult to determine since some *rha* replication is already seen during the first pulse. However, the bulk of the *rha* replication follows during the next 4 min of replication at both growth rates used. Since *ilv* and *rha* are separated by only 2 min on the *E. coli* map (21), we feel that the origin of replication must lie very near *ilv* and possibly between *dnaA* and *ilv* (Fig. 5).

Rate of travel of replication forks. The results presented above clearly show a difference in the time of replication of late markers λ and *thr* at the two growth rates used. Using the data obtained, we estimated the times required to replicate the chromosome as 40 min in Casamino Acids medium and 52 min in glucose minimal medium. The value of 40 min for Casamino Acids is in agreement with the model of Cooper and Helmstetter (8) and with recent results obtained with exponential cultures of *E. coli* K-12 (M. Chandler, personal communication). The time of 52 min for minimal medium is longer than expected from the Cooper-Helm-

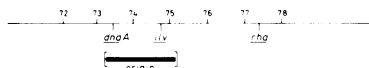


FIG. 5. *E. coli* genetic map in the region of the chromosome origin. The map positions shown are from the standard *E. coli* map (21).

stetter model, which predicts a constant chromosome replication time of 41 min at 37 C for generation times of less than 60 min, and from Chandler's unpublished data. Nevertheless, we feel that the difference observed in our experiments is real; however, it should be noted that these cultures have been starved first for amino acids and subsequently for thymine, and the rate of fork travel may be altered as a result.

The spread in replication of late markers suggests a large variation in the rate of travel of a replication fork. The marker *ilv* is replicated over a very short period, showing that initiation occurs synchronously. Hence, the spread for late markers seems to be derived from variation in the rate at which forks move and not from asynchronous initiation. This variation is large; however, the same variation has been seen for exponential (18) and synchronized cultures (2).

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LITERATURE CITED

- Adams, M. H. 1959. Bacteriophages. Interscience Publishers, Inc., New York.
- Bird, R., and K. G. Lark. 1968. Initiation and termination of DNA replication after amino acid starvation of *E. coli* 15T. Cold Spring Harbor Symp. Quant. Biol. **33**:799-808.
- Bird, R., J. Louarn, J. Martuscelli, and L. G. Caro. 1972. The origin and sequence of chromosome replication in *Escherichia coli*. J. Mol. Biol. **70**:549-566.
- Brunk, C. F., and V. Leick. 1969. Rapid equilibrium isopycnic CsCl gradients. Biochim. Biophys. Acta **179**:136-144.
- Burton, K. 1956. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochemistry **62**:315-323.
- Caro, L. G., and C. M. Berg. 1968. Chromosome replication in some K12 strains of *Escherichia coli*. Cold Spring Harbor Symp. Quant. Biol. **33**:559-573.
- Caro, L. G., and C. M. Berg. 1969. Chromosome replication in *Escherichia coli*. II. Origin of replication in F⁻ and F⁺ strains. J. Mol. Biol. **45**:325-336.
- Cooper, S., and C. E. Helmstetter. 1968. Chromosome replication and the division cycle of *Escherichia coli* B/r. J. Mol. Biol. **31**:519-540.
- Helmstetter, C. E. 1968. Origin and sequence of chromosome replication in *Escherichia coli* B/r. J. Bacteriol. **95**:1634-1641.

10. Hohlfeld, R., and W. Vielmetter. 1973. Bidirectional growth of the *E. coli* chromosome. *Nature N. Biol.* **242**:130-132.
11. Jacob, F., and A. Campbell. 1959. Sur le système de répression assurant l'immunité chez les bactéries lysogènes. *C. R. Acad. Sci. (Paris)* **248**:3219-3221.
12. Jacobson, M. K., and K. G. Lark. 1973. DNA replication in *Escherichia coli*: evidence for two classes of small deoxyribonucleotide chains. *J. Mol. Biol.* **73**:371-396.
13. Jonasson, J. 1973. Evidence for bidirectional chromosome replication in *Escherichia coli* C based on marker-frequency analysis by DNA-DNA hybridization with P2 and λ prophages. *Mol. Gen. Genet.* **120**:69-90.
14. Kourilsky, Ph., J. Leidner, and G. Y. Tremblay. 1971. DNA-DNA hybridization on filters at low temperature in the presence of formamide or urea. *Biochimie* **53**:1111-1114.
15. Lark, K. G., T. Repko, and E. J. Hoffman. 1963. The effect of amino acid deprivation on subsequent deoxyribonucleic acid replication. *Biochim. Biophys. Acta* **76**:9-24.
16. Maaløe, O., and P. C. Hanawalt. 1961. Thymine deficiency and the normal DNA replication cycle I. *J. Mol. Biol.* **3**:144-155.
17. Masters, M., and P. Broda. 1971. Evidence for the bidirectional replication of the *Escherichia coli* chromosome. *Nature N. Biol.* **232**:137-140.
18. Nagata, T., and M. Meselson. 1968. Periodic replication of DNA in steadily growing *Escherichia coli*: the localized origin of replication. *Cold Spring Harbor Symp. Quant. Biol.* **33**:553-557.
19. Schubach, W. H., J. D. Whitmer, and C. I. Davern. 1973. Genetic control of DNA initiation in *Escherichia coli*. *J. Mol. Biol.* **74**:205-221.
20. Taylor, A. L. 1963. Bacteriophage-induced mutation in *Escherichia coli*. *Proc. Nat. Acad. Sci. U.S.A.* **50**:1043-1051.
21. Taylor, A. L., and C. D. Trotter. 1972. Linkage map of *Escherichia coli* strain K-12. *Bacteriol. Rev.* **36**:504-524.
22. Wolf, B., A. Newman, and D. A. Glaser. 1968. On the origin and direction of replication of the *Escherichia coli* K-12 chromosome. *J. Mol. Biol.* **32**:611-629.
23. Wolf, B., M. G. Pato, C. B. Ward, and D. A. Glaser. 1968. On the origin and direction of replication of the *E. coli* chromosome. *Cold Spring Harbor Symp. Quant. Biol.* **33**:575-584.
24. Yahara, I. 1971. On the origin of replication of *Escherichia coli* chromosome. *J. Mol. Biol.* **57**:373-376.