*THE ORIGIN AND DIRECTION OF REPLICATION OF THE CHROMOSOME OF ESCHERICHIA COLI B/r**

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In the resting state between replication cycles, the chromosome of *E. coli* is a simple circular unit (probably a single molecule of double-stranded DNA about 1 mm in length).[1] *E. coli* grown slowly in minimal medium possesses chromosomes which replicate by the continuous linear motion of a single Y-shaped growing point that progresses along the molecule at approximately constant speed. Numerous attempts have been made to determine the direction of this replication process along the genetic map and the origin from which it begins, but contradictory results have been reported.

Some investigators[2-5] find that the origin is located at the integration site of the F factor in Hfr strains, but there is disagreement about the direction of replication. No unique origin for F^- strains is found by some authors.[2, 4]

Other investigators[6-8] have found a single origin for several Hfr and F^- strains at a position corresponding to the 7–8 o'clock region of the genetic map.[1] Evidence has been presented for a unique, though undetermined, origin for F^- strains,[10] as well as evidence for the noninvolvement of the F factor in determining the origin of replication.[11]

These differences may reflect differences among the strains tested or they may arise from technical differences among the methods used. The present experiments were done to locate the origin and direction of replication in *E. coli* B/r by techniques chosen in an attempt to avoid some of the artifacts of earlier methods.

Materials and Methods.—Bacterial strain: *E. coli* B/r (ATCC 12407) was used throughout.

Media: Cells were routinely grown on a minimal salts medium that consisted of two parts which were mixed shortly before use. The first solution contained 2.0 gm $(NH_4)_2SO_4$, 6.0 gm Na_2HPO_4, 3.0 gm KH_2PO_4, 3.9 gm NaCl, and 0.011 gm Na_2SO_4 dissolved in deionized H_2O; the second part contained 0.2 gm $MgCl_2$, 0.1 gm $CaCl_2$, and 0.0005 gm $FeCl_3 \cdot 7H_2O$ dissolved in 800 ml deionized H_2O. The carbon source was either glucose (0.2%) or glycerol (0.2%), depending upon the experiment.

Experimental Procedures.—Two different techniques were used to determine when a particular gene replicates during the division cycle of the cell. In technique A, newly divided cells were separated physically from an exponentially growing culture in order to obtain a synchronized culture. Samples taken at various times from this synchronized culture were used to measure the "inducibility" of several enzymes as a function of cell age. "Inducibility" is defined as the amount of enzyme synthesized in a fixed, short period of induction. The synchrony procedure used[12, 13] permits one to collect the newly divided cells that have been eluted from a membrane filter to which a growing population of bacteria have been affixed. When necessary, cells were concentrated by collecting newly divided cells in chilled flasks for an hour, filtering the chilled cells, and resuspending in a desired volume of warm conditioned medium, i.e., medium that had sustained growth to about 3×10^7 cells/ml and had been filtered to remove the bacteria. Aliquots were taken from synchronously growing cultures and added to tubes containing appropriate inducers. After 10 min of induction, chloramphenicol was added to a final concentration of 100 μg/ml to inhibit further protein synthesis and the samples were chilled until assayed.

In technique B,[14] induction of the enzymes was carried out in exponentially growing cultures. The cells were then separated according to their ages in the exponential culture, and the amount of enzyme/cell was determined. Separation was accomplished by affixing the cells to a membrane filter and continuously collecting the eluted cells. The first cells eluted off the membrane were the progeny of the oldest cells in the exponential population, while cells eluted later were the progeny of younger cells in the original culture. It is believed that one of the progeny of a cell dividing on the membrane remains affixed, and therefore the elution pattern repeats after one generation as the progeny of the oldest cells in the original population undergo division. Exponential cultures were induced for enzyme production for 3 min, the cells were bound to the membrane and washed to remove inducer, and elution was carried out with the conditioned medium. Three-min samples were collected continuously and the number of eluted cells and the amount of enzyme per cell determined.

Enzyme induction: The enzymes and concentrations of inducers used were: β-galactosidase, 10^{-3} *M* isopropyl-1-thio-β-D-galactoside (IPTG); tryptophanase, 0.5 mg/ml L-tryptophan; and D-serine deaminase, 0.2 mg/ml D-serine. Glycine (0.2 mg/ml) was added with the D-serine to overcome the toxic effects of the serine.

Enzyme assays: The assay for β-galactosidase was that of Abbo and Pardee;[15] the tryptophanase assay, a modification of the assay of Gartner and Riley;[16] and the assay for D-serine deaminase, that of Pardee and Prestidge.[17]

Measurement of growth and synchrony: Cell numbers were measured with a Coulter counter equipped with a 30-μ orifice. The counter was modified with a Nuclear Data pulse-height analyzer to allow observation of the size distribution of the sample being counted. This modification allowed rapid monitoring of the quality of synchrony.

Results and Conclusions.—Enzyme synthesis with the use of technique A: Aliquots were taken from a synchronously growing culture in glucose medium and induced for the synthesis of β-galactosidase. Figure 1 shows the amount of enzyme present at the end of the ten-minute induction periods during two generations of synchronous growth. The enzyme is inducible at all times in the cycle,

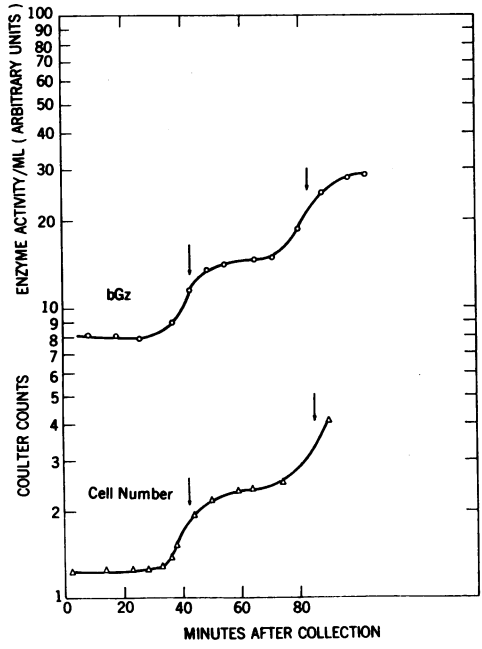


FIG. 1.—Cell numbers and β-galactosidase (*bGz*) inducibility for a glucose-grown culture synchronized by technique A. The arrows indicate the mid-points of the rise for each step.

and the inducibility rises by a factor of 2 at about the time of division. The mid-point of the rise of inducibility is indicated by an arrow.

If the doubling of inducibility is due to the replication of the lactose genes, then other enzymes should show steps in inducibility at other times in the cycle consistent with their map locations. To allow measurement of enzymes other than β-galactosidase, the cells were concentrated as described above. The concentration procedure did not affect the position of the step of inducibility for β-galactosidase, and Dr. D. J. Clark in this laboratory showed that the position of the start of new rounds of DNA synthesis was unaltered (unpublished data). Figure 2*A* shows the inducibility for β-galactosidase and D-serine deaminase in glucose

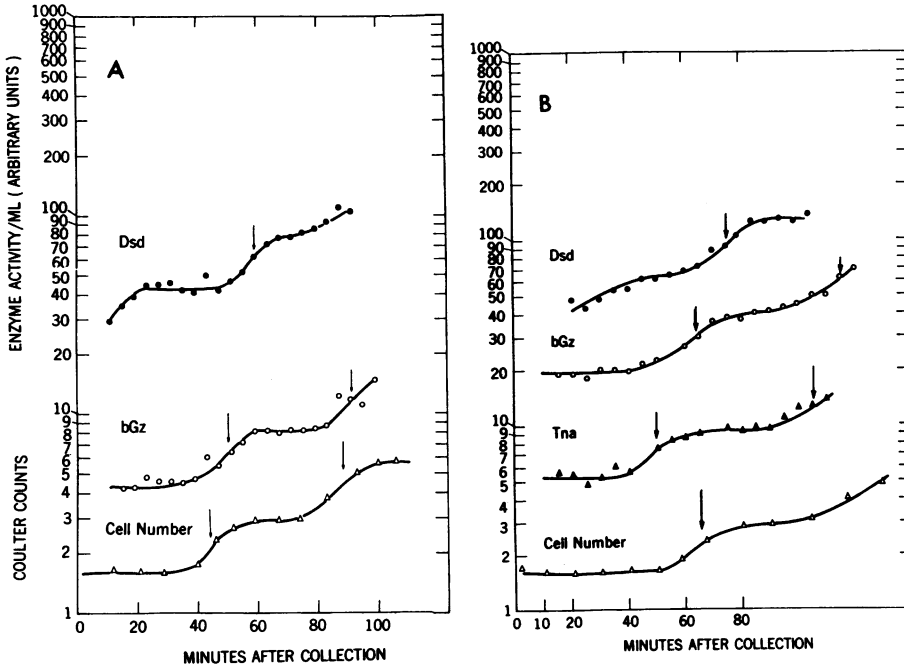


FIG. 2.—Cell numbers and enzyme inducibility for cultures synchronized by technique A and concentrated as described. (*A*) Glucose-grown cells; (*B*) glycerol-grown cells. The arrows for the second generation for bGz and Tna are drawn at a point indicating a rise above the previous plateau equivalent to the rise between the first and second plateaus. Other experiments carried out for longer times have shown plateaus in the third generation at the expected positions.

● Dsd, D-serine deaminase; ○ bGz, β-galactosidase; ▲ Tna, tryptophanase.

cultures, and Figure 2*B* shows the results for β-galactosidase, D-serine deaminase, and tryptophanase in glycerol cultures. Tryptophanase was not measured in glucose cultures because of the reduction in enzyme levels caused by catabolite repression.

Enzyme synthesis with the use of technique B: Exponential cultures were induced for β-galactosidase synthesis. Figure 3*A* and *B* shows the number of eluted cells and the amount of β-galactosidase per cell in each sample for glucose

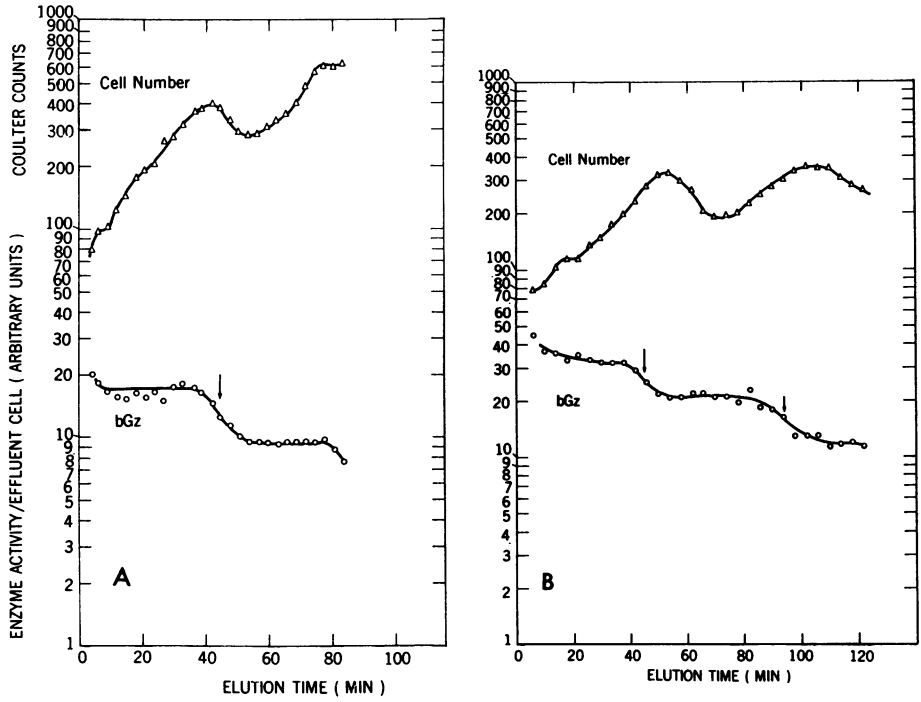


FIG. 3.—Amount of β-galactosidase per cell and number of cells per sample for cultures treated as described for technique B. (*A*) Glucose-grown cells; (*B*) glycerol-grown cells.

and glycerol, respectively. The position of the twofold steps in enzyme/cell shown in Figure 3 indicates the place in the life cycle that the inducibility for β-galactosidase doubled. Cells eluted before the time of the step, i.e., the progeny of cells past that stage of the cycle at the time of induction, have twice the amount of enzyme than have the cells eluted after the time of the step. The pattern is repeated in the second generation.

The effect of inhibition of DNA synthesis on inducibility: The assumption that the replication of a gene will cause a doubling of the inducibility of the corresponding enzyme is used throughout this work. Evidence favoring this assumption can be found in many sources.[10] The assumption predicts that the inhibition of DNA synthesis before the replication of a gene will abolish the step of inducibility for the corresponding enzyme. This prediction is verified as shown in Figure 4. Fluorouracil deoxyribose, 10 μg/ml, an inhibitor of DNA synthesis, abolished the step of inducibility for β-galactosidase when added before the normal appearance of the step. Similar results were obtained with 10 μg/ml nalidixic acid, another inhibitor of DNA synthesis.

Location of the origin and direction of replication: The locations of the inducibility steps for glycerol cultures are summarized in Figure 5, and the locations of the corresponding structural genes are shown on the genetic map.[9] The time in the cycle that new rounds of DNA synthesis are initiated, 20–25 minutes before division, is indicated as SOR (start of rounds).[13]

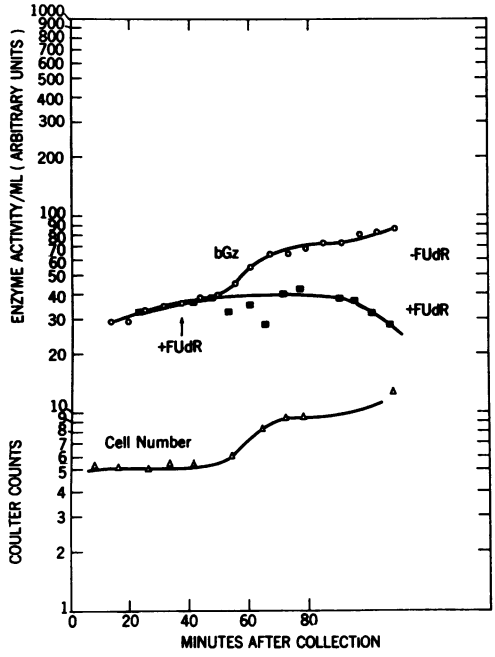
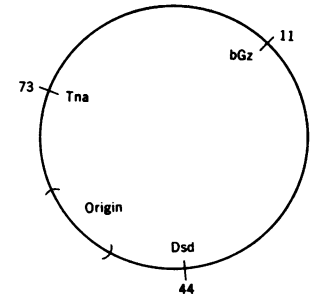


FIG. 4.—The effect of fluorodeoxyuridine (*FUdR*), an inhibitor of DNA synthesis, on β-galactosidase inducibility in a glycerol-grown culture synchronized by technique A. The inhibitor was added to half the culture at 37 min.

The order of replication of the genes . . .TbDTb. . . is consistent only with a clockwise direction of replication. By correlating the intervals shown in Figure 5 with the locations of the relevant genes on the genetic map, the origin can be placed between 7 and 8 o'clock on the genetic map. The data from glucose cultures show the same results.

If no data were available for the location of the start of new rounds of DNA synthesis, the enzyme data from the glycerol culture alone, with the use of technique A, would predict that the start of rounds occurs between the steps of inducibility for D-serine deaminase and tryptophanase. The interval between these two steps is longer than predicted from their map locations and includes the period in the cycle when no DNA synthesis is taking place. The gap in DNA synthesis is estimated to be about ten minutes in duration for glycerol-grown cultures.[14]

When the times of the steps for β-galactosidase with the use of technique B are correlated with the times for the start of rounds of DNA synthesis with the use of the same technique, the origin can be determined and agrees with the origin determined with the use of technique A. The start of rounds for glucose cultures occurs about 20–25 minutes after the peak of eluted cells and for glycerol cultures about 10 minutes after the peak.[14] Figure 3 shows that the enzyme step appears about two minutes after the peak of eluted cells for glucose cells and about ten minutes before the peak for glycerol cells. Both cultures show about a 20-minute interval between the start of rounds and the step for β-galactosidase, and since the chromosome replicates in about 40 minutes, this places the origin halfway around the chromosome from the lactose genes.



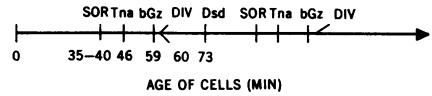


FIG. 5.—A summary of the data for the inducibility of three enzymes in glycerol-grown cells synchronized by technique A, and the location of the structural genes for the enzymes on the genetic map.[9] The approximate location of the origin is indicated.

Discussion.—The origin found in this work and that found by Helmstetter (unpublished results), who has studied several enzymes with the use of technique B, agree with the results of Wolf *et al.*, Abe and Tomizawa, and Cerda-Olmeda *et al.* These three experiments required the use of amino acid starvation and/or the incorporation of bromouracil. Because these were nonphysiological treatments, the argument has been made that an artificial origin was observed and that the correct model for normal vegetative growth is that of Nagata.[2] The fact that the same origin is found in the experiments reported in this paper makes this argument much less plausible.

Some of the differences found in the literature may be due to the strains used. However, it is likely that differences are also due to the techniques used. For example, cells synchronized by the technique used by Nagata[2] show an exponential increase in the rate of DNA synthesis during the cell cycle[3] as opposed to the constant rate of synthesis with a doubling of rate around the middle of the cycle found for the synchrony technique in technique A.[13]

A finding of Wolf *et al.*[7] that some Hfr strains may show more than one origin suggests that a vegetative origin may exist (as defined in this paper) as well as various possible alternate origins, perhaps identical with the possible sites of integration of F factors. Artificially shocking the cells may trigger the mating origin in Hfr strains and cause initiation at one or more of the possible F integration sites in F^- strains which have no integrated F factor to determine a unique alternate site. In some strains the mating origin may function even during normal vegetative growth.

Summary.—The origin and direction of replication of the chromosome of *E. coli* B/r have been determined. The origin is located at a position corresponding to the 7–8 o'clock region of the genetic map, and the direction of replication is clockwise.

Note added in proof: The results of Helmstetter, now in print (*J. Bacteriol.*, **95**, 1634 (1968)), are in good agreement with those described above, obtained with the use of technique B.

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