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Symmetric Replication of the *Bacillus subtilis* Chromosome*

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Abstract. Synchronously replicating chromosomes in germinating spores of *Bacillus subtilis* were labeled near the origin with [^{3}H]bromouracil. The label appeared in heavy-light DNA. When cell growth and chromosome replication were continued in unlabeled bromouracil, nearly all the tritium label was transferred to heavy-heavy DNA while the terminus remained unreplicated. This implies that both origins of the replicating chromosome can undergo reinitiation. Therefore, during multifork replication the chromosome takes on the symmetric "dichotomous" form rather than the asymmetric configuration predicted by the rolling circle model.

It has been unclear for some time whether the two template strands of the bacterial chromosome are functionally distinguishable during replication. The classical model[1,2] assumes identical functions for the two parental strands, hence a "symmetric" replication process. In the alternative, "rolling circle" model, elaborated in detail by Gilbert and Dressler[3] and by Eisen *et al.*,[4] one template strand remains circular throughout the replication of the chromosome, while the other is continually displaced around the circle by new strand elongation, and then serves as a template for the other new strand. The two strands have different functions; replication is "asymmetric."

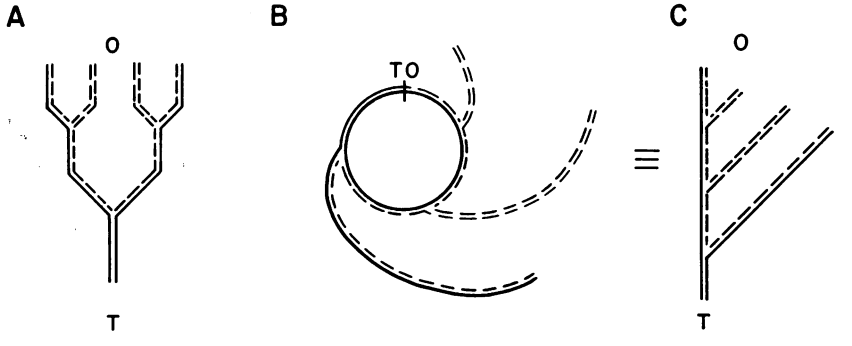

FIG. 1. (*A*) Dichotomous chromosome configuration[5,6] based on symmetric replication. (*B*) Multifork replication in the rolling circle model.[3] If the chromosome is schematically made linear (as Fig. 1*A*), it gives the asymmetric configuration 1*C*. (*O*, replication origin; *T*, terminus).

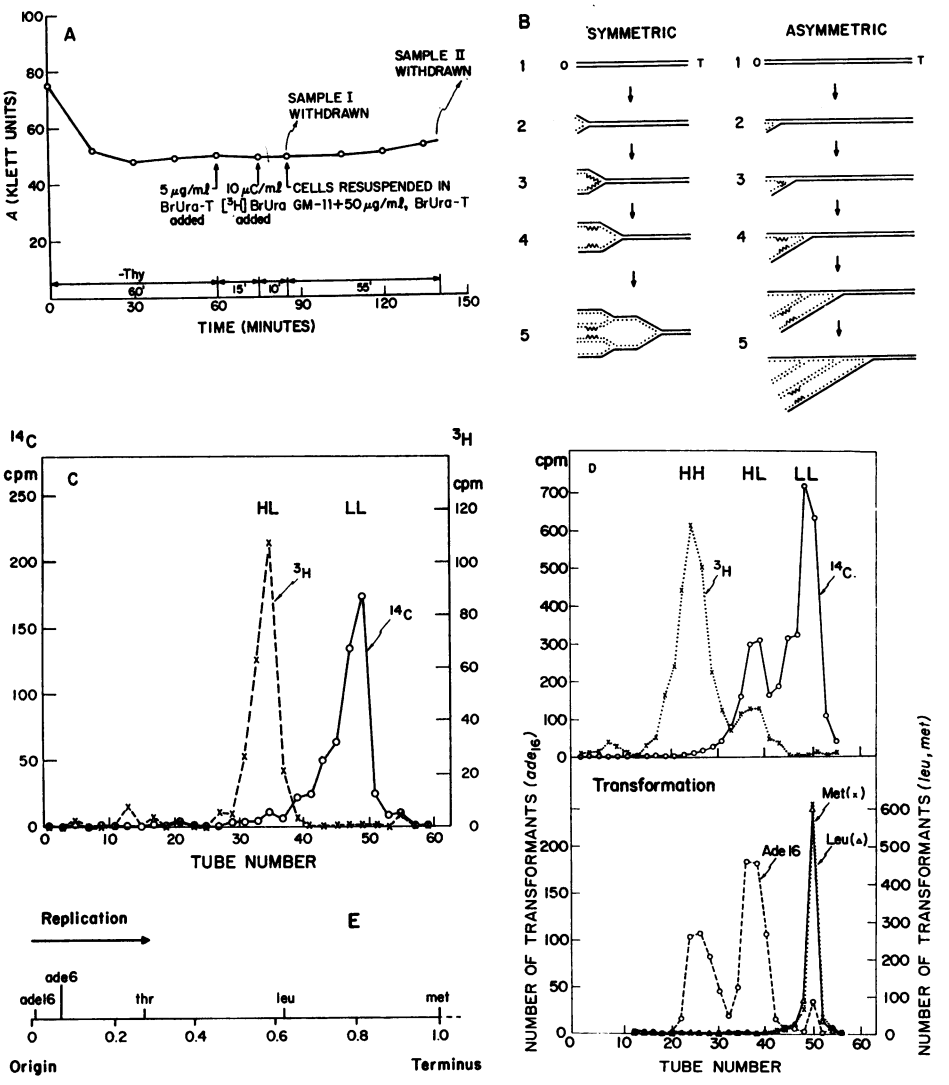

FIG. 2A. Germination curve and labeling protocol for experiment. Spores of *B. subtilis* with uniform [^{14}C]DNA label were germinated in 40 ml of medium GM-11 (without thymine) plus 20 μg/ml uracil. After 60 min, a 50:1 mixture of 5-bromouracil and thymine (50:1 BrUra–T) was added to a concentration of 5 μg/ml. At 75 min, [^{3}H]BrUra was added (0.2 μg/ml, 10 μCi/ml), and the germinating spores were labeled for 10 min. At 85 min, 10 ml was removed (*Sample I*); the rest of the cells were collected on a 47 mm Millipore HAWP filter, washed with 50 ml of GM-11 medium[10] containing 20 μg/ml uracil, and resuspended in 30 ml of GM-11 containing 20 μCi/ml uracil and 50 μg/ml unlabeled 50:1 BrUra–T (all media 37°C). At 140 min incubation was stopped (*Sample II*).

(*B*) Schematic presentation of chromosome configurations, showing labeling patterns of the germinating spores in the experiment above, for both the symmetric and asymmetric models. (1) During the thymine-starvation period; (2) after 15 min growth in unlabeled 50:1 BrUra–T; (3) after labeling with [^{3}H]BrUra; (4) during continued replication in unlabeled BrUra–T medium; (5) after reinitiation and continued replication in BrUra–T medium (– – –, ^{14}C-labeled parental DNA; · · ·, BrUra DNA; ∿∿, ^{3}H-labeled BrUra DNA).

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In chromosomes with one replication point, the differences between the two models are in detail, and an experimental choice between them is difficult. However, the occurrence in bacteria of multifork replication[5-8] (for example, at high growth rates) provides a straightforward means of deciding the question. The rolling circle model predicts that in the replicating chromosome only one of the origins is capable of reinitiation (Fig. 1*C*). Symmetric replication implies a "dichotomous"[5,6] configuration in the multifork chromosome (Fig. 1*A*); reinitiation can take place at both origins. Here it will be shown that both origins of the chromosome undergo reinitiation.

Materials and Methods. *B. subtilis* 23 *thy, his,* But-1310,[9] a mutant capable of sustained growth in medium containing 50:1 bromouracil–thymine (50:1 weight ratio = 29:1 molar ratio) was used throughout these experiments. Spores with uniformly ^{14}C-labeled DNA were prepared by overnight culture in GM-11,[10,11] a synthetic spore-germination medium, supplemented with [^{14}C]thymine (5 μg/ml; 540 μCi/μmol) and 20 μg/ml unlabeled uracil, followed by growth and sporulation for one week on plates of tryptose–blood–agar base (Difco) supplemented with [^{14}C]thymine (10 μg/ml, 16 μCi/μmol) plus 0.5% agar. Spores were harvested and purified by treatment with lysozyme and sodium dodecyl sulfate.[12]

Results. Fig. 2*A* shows the experimental procedure for labeling the newly synthesized strands near the origin with [^{3}H]BrUra, then allowing continued growth and replication in medium containing 50:1 bromouracil–thymine (50:1 BrUra–T) while reinitiation takes place. The chromosome configurations associated with each stage of growth are shown in Fig. 2*B* according to both the symmetric and asymmetric models for replication.

The chromosomes of *B. subtilis* spores are strictly aligned, without replication forks.[5] During spore germination, replication is synchronous, beginning at the origin.[6] In this experiment, because cell growth in medium GM-11 is too slow to ensure multifork replication under normal conditions, and because cells are lysed with difficulty during the early phases of germination, the spores were germinated for 60 min in GM-11 plus 20 μg/ml uracil without added thymine or bromouracil.

(*C*) DNA density profile immediately after ^{3}H-labeling. Sample I (Fig. 2*A*) was heated to 70°C for 15 min and centrifuged. The cells were resuspended in 2 ml of a solution of 0.1 M EDTA (pH 7.6)–0.5 M sucrose–3 mg/ml lysozyme and incubated for 45 min at 37°C. 0.2 ml of 20% sodium dodecyl sulfate was added and the solution incubated for 15 additional minutes. 1.86 ml of the lysate was shaken with 2.40 g of CsCl (E. Merck, optical grade). 1.5 ml of the clear solution was used as the middle layer of a CsCl step gradient; the top and bottom layer were 1.5-ml CsCl solutions (d = 1.646 and d = 1.765 respectively) in 0.01 M Tris pH 8.0–0.001 M EDTA. After 35 hr centrifugation at 35,000 rpm (25°C) in a Spinco SW 39 rotor, two-drop fractions were collected, precipitated, and counted as described previously.[10] O, ^{14}C; ×, ^{3}H; H, heavy; L, light.

(*D*) Upper chart: DNA density profile and labeling pattern in multifork chromosomes. Sample II (Fig. 2*A*) was killed, lysed, and centrifuged, and alternate fractions were counted by the methods in Fig. 2*C*. 83% of the tritium label appears in the heavy-heavy peak. Lower chart: transforming activity of DNA in the above gradient. Alternate fractions of this gradient were collected in sterile capped tubes and diluted with 1 ml of sterile standard saline citrate solution.[10] 0.2 ml of each fraction was used to transform a triple mutant, *B. subtilis* 168 *leu, met,* ade_{16} (Mu8u5u16), by the method of Bott and Wilson.[24] *Leu-* and *met-* transforming DNA is present only in the light-light, parental peak; the first replication point has not reached either of these loci in any cells.

(*E*) Map of the *B. subtilis* chromosome, showing locations of the markers used in this experiment.[2,18]

This allows accumulation of the "initiation protein,"[13-15] facilitates cell lysis, and also permits synchronous replication when bromouracil and thymine are added (Fig. 2*B*, stage 1). There was no loss in cell viability in these experiments either during or after the thymine starvation period.

If [^{3}H]BrUra is added immediately after the thymine-starvation period, much of the label appears in light-light rather than hybrid DNA (Quinn and Sueoka, unpublished). This is presumably due either to a period of DNA repair after the thymine starvation[16,17] or to the covalent connection of new-strand origins to the termini of parental strands.[18,19] To avoid this complication, we added 5 μg/ml of 50:1 BrUra–T to the germination medium 15 min before the labeling period (Fig. 2*B*, stage 2); for the purposes of this experiment it is necessary only to label the new strands near the daughter origins, not at them.

After a 10 min period of labeling with [^{3}H]BrUra, a 10-ml sample was withdrawn (Fig. 2*B*, stage 3). The cesium-chloride density profile of the DNA is shown in Fig. 2*C*. Virtually all the label appears in heavy-light DNA; there is no incorporation for repair, and essentially no reinitiation has occurred before or during the labeling period. The ^{14}C parental DNA profile shows that 6% of the chromosome has replicated.

The remaining cells (30 ml) were then resuspended in GM-11 plus 50 μg/ml 50:1 BrUra–T and 20 μg/ml uracil and allowed to continue growth and replication for an additional 55 min (stage 5). The cesium-chloride profile of the DNA is shown in Fig. 2*D*.

Fig. 2*B*, stage 5, shows the chromosome configurations and labeling patterns to be expected with multifork replication for each model. In the asymmetric case, no matter how many rounds of reinitiation take place, no more than half the tritium will be transferred to heavy-heavy DNA. Until the first replication fork reaches the terminus, one daughter origin cannot be replicated again, and its label remains in the hybrid DNA peak. In the symmetric model, on the other hand, all the tritium label can be transferred. If synchrony is very good and reinitiation occurs on every chromosome, 100% of the label will appear in the heavy-heavy peak.

Fig. 2*D* shows 83% of the tritium label in the heavy peak. The label remaining in the hybrid peak is probably the result of incomplete synchrony in reinitiation, as will be shown below. Fig. 2*D* (lower chart) shows the transforming activity of DNA in this gradient for three markers, ade_{16}, *leu*, and *met*[2,19] (Fig. 2*E*). The profile shows that all DNA from the ade_{16} locus near the origin has undergone one round of replication, and that 86% has been replicated twice. This suggests that reinitiation has occurred in most but not all chromosomes.

To prove that the tritium in the heavy-heavy peak results from reinitiation, rather than from the beginning of a second cycle of replication, it is necessary to show that no chromosomes have completed the first round. In Fig. 2*D*, 32% of the ^{14}C-label is in hybrid DNA, which indicates that on the average, the first replication fork has travelled a third of the distance from the origin to the terminus. More important, *leu* and *met* transforming activities occur only in the light-light, parental peak. None of the chromosomes have even approached

completion. This evidence is much stronger than the ^{14}C density-transfer data; it is not weakened by the possibility of asynchronous replication. Therefore the fact that nearly all the labeled origins reinitiated *must* be a consequence of dichotomous replication; it cannot have resulted from some chromosomes having completed their replication and begun a new cycle in the asymmetric model.

Fig. 3 shows the results of a similar experiment (Figs. 3*A* and 3*B* correspond to Figs. 2*C* and 2*D* respectively). In this case, chromosome replication in unlabeled 50:1 BrUra–T was allowed to continue for an additional 25 min; the ^{14}C-profile (Fig. 3*B*) shows that the first replication fork has travelled halfway down the chromosome. Here 88% of the tritium label appears in heavy-heavy DNA. Again this label distribution cannot have resulted from asym-

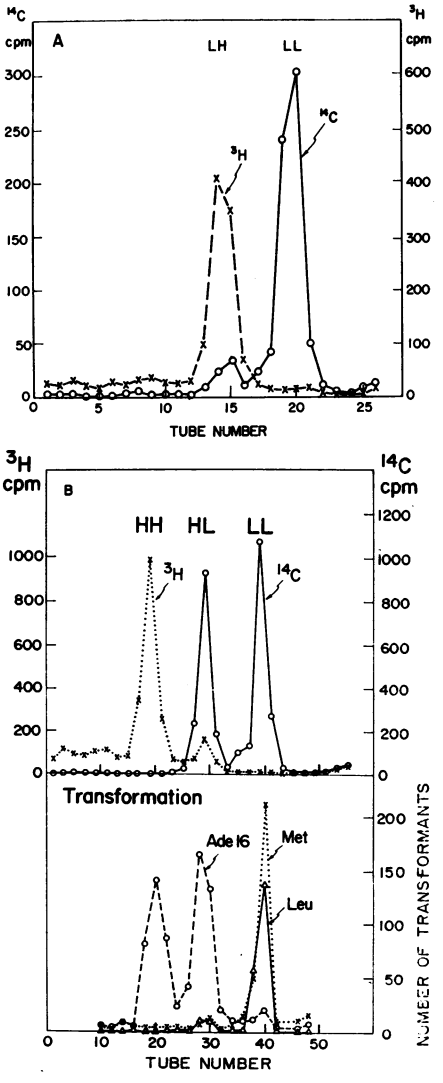

FIG. 3. Experiment similar to that in Fig. 2, with longer chase period. Spores were germinated and tritium-labeled by the procedure in Fig. 2 with the following modifications: (*a*) Spores were germinated in thymineless medium for 65 min rather than for 60. (*b*) After resuspension at 90 min, cells were grown for 80 min rather than 55 in the unlabeled 50:1 BrUra–T medium. (*c*) For each sample, the lysis solution was doubled in volume and shaken with 4.80 g of CsCl. 3.5 ml of this solution was used to form the entire CsCl gradient; it was spun on a Spinco SW 50.1 rotor (at 25°C) for 15 hr at 40,000 rpm, followed by 80 hr at 35,000 rpm. For sample I (taken at 90 min, immediately after ^3H-labeling) four-drop fractions were collected and counted. Fig. 3*A* shows that the tritium was incorporated entirely into heavy-light DNA. For sample II (170 min), two-drop fractions were collected and alternate ones were counted (Fig. 3*B*, upper chart) or used for transformation (Fig. 3*B*, lower chart). In this sample, 88% of the tritium label (above background) is in heavy-heavy DNA; replication of late markers is less than 5%.

TABLE 1. *Quantitative transfer data.*

Samples	% ^{14}C in HL	% ^{3}H in HL	% ^{3}H in HH	% ade_{16} in LL	% ade_{16} in HL	% ade_{16} in HH	% *met* in LL
			Experiment A (Fig. 2)				
I	6	100	0	...	...	...	...
II	32	17	83	5	57	38	100
			Experiment B (Fig. 3)				
I	11	100	0	...	...	...	...
II	46	12	88	5	52	43	>95

H, heavy; L, light.

metric chromosome replication. Quantitative data from Figs. 2 and 3 are summarized in Table 1.

Discussion. Several previous investigations have favored the symmetric over the asymmetric model in bacteria.[6,20-22] However, models of rolling-circle replication can be constructed which would give rise to the results of these experiments. The advantages of the method reported here are its directness and its lack of reliance on any assumptions either about the number and timing of reinitiations or about the distribution of chromosomes at different growth stages among cells. This proof of symmetric replication provides a sound theoretical basis for our interpretation[5,6] and detailed quantitative predictions[23] from previous experimental results.

During multiforked chromosome replication, both origins may undergo reinitiation, and the chromosome attains the "dichotomous" configuration. To what extent the functional equivalence of template strands persists at the molecular level is, of course, unclear. However, the rolling circle model of replication does not hold true in *B. subtilis*, and chromosome replication in the gross "topological" sense is symmetric.

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Abbreviations: BrUra, 5-bromouracil; H heavy, L light DNA.

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