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42. We thank members of each laboratory for helpful discussions; C. Boone, R. A. Arkowitz, J. Chant, D. Lew, M. Funk, E. Elion, E. O'Shea, H.-O. Park, E. Bi, and E. Leberer for plasmids and strains; B. Catarin for polyclonal antibodies to Far1p; and E. Leberer for antibodies to Bem1p. We also thank N. Valtz, S. Henchoz and K. Peter for help during early aspects of this work and J. Philips, V. Simanis, and R. Iggo for critical reading of the manuscript. L.S.H. was supported by an NIH postdoctoral fellowship. Work in the I.H. laboratory was supported by an NIH research grant (GM48052). P.M.P. is supported by grants from the Worcester Foundation, the Millipore Foundation, and NIH (GM57769). M.P. is supported by the Swiss National Science Foundation, the Swiss Cancer League, and a Helmut Horten Incentive award. I.H. dedicates this paper to J. Stahl.

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# Localization of Bacterial DNA Polymerase: Evidence for a Factory Model of Replication

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**Two general models have been proposed for DNA replication. In one model, DNA polymerase moves along the DNA (like a train on a track); in the other model, the polymerase is stationary (like a factory), and DNA is pulled through. To distinguish between these models, we visualized DNA polymerase of the bacterium *Bacillus subtilis* in living cells by the creation of a fusion protein containing the catalytic subunit (PolC) and green fluorescent protein (GFP). PolC-GFP was localized at discrete intracellular positions, predominantly at or near midcell, rather than being distributed randomly. These results suggest that the polymerase is anchored in place and thus support the model in which the DNA template moves through the polymerase.**

For all organisms, the production of viable progeny depends on the faithful replication of DNA by DNA polymerase. A conceptual question about in vivo DNA replication remains unsettled. During replication, does the DNA polymerase move along the DNA template? or is the DNA polymerase in a fixed position with the DNA template moving through the replication machinery? Studies with eukaryotic cells have indicated that DNA replication proteins and newly replicated DNA are present at numerous discrete foci [so-called replication factories (*1*)], resulting in the hypothesis that DNA replication occurs at fixed locations. However, analysis of eukaryotic DNA polymerase is complicated because replication starts from many different origins and because it is difficult to orient the foci within the eukaryotic nucleus.

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Like many bacteria, *Bacillus subtilis* has a single circular chromosome [~4200 kilobase pairs (*2*)], and DNA replication initiates from a single origin (*oriC*) and proceeds bidirectionally (*3*). Most of the proteins present at the replication fork are conserved in prokaryotes and eukaryotes (*4*).

We attempted to determine whether, in a population of cells at different stages of the replication cycle, the replicative DNA polymerase of *B. subtilis* functions at fixed intracellular positions or if it is randomly distributed along the nucleoid. We visualized DNA polymerase in living cells using a fusion protein consisting of the catalytic subunit (PolC) attached in-frame to green fluorescent protein (GFP). *polC-gfp* was placed in single copy in the *B. subtilis* chromosome under control of the endogenous *polC* promoter (*5*). PolC-GFP supported DNA replication and cell growth when it was present as the only source of the catalytic subunit, and it was visible as discrete fluorescent foci, at or near midcell, in most cells during exponential growth (Fig. 1A) (*6*). In these cells, the DNA occupied most of the cytoplasmic space and appeared to extend to the cell boundaries (Fig. 1B).

Two experimental observations indicated that the foci correspond to DNA polymerase at replication forks: (i) the presence of foci was dependent on continued DNA synthesis and (ii) the number of foci per cell increased at faster growth rates. We prevented reinitiation of DNA replication by inhibiting expression of DnaA, which binds to *oriC* and is required for assembly of the replication complex (*3, 4*). We fused *dnaA* to the LacI-repressible isopropyl-β-D-thiogalactopyranoside (IPTG)–inducible promoter Pspac (*7*) so that transcription of *dnaA* was IPTG-dependent. In the presence of IPTG (expression of *dnaA*), <5% of the cells lacked the visible

**Table 1.** The number of PolC-GFP foci per cell increased at faster growth rates. Cells were grown at 30°C in defined minimal medium (*21*) with the indicated carbon source (1%). All 20 amino acids (aa) were added in glucose + aa. Doubling times were rounded to the nearest 5 min, and samples were taken during exponential growth. In succinate, only one cell had three foci, and no cells had four. Six cells had five foci of PolC-GFP (three each from the glucose and glucose + aa cultures).

| Doubling time (min) | Supplement | Total cells (*n*) | Cells with indicated number of foci (%) | | | | |
|---|---|---|---|---|---|---|---|
| | | | 0 | 1 | 2 | 3 | 4 |
| 230 | Succinate | 1317 | 24 | 56 | 19 | 0.08 | <0.08 |
| 115 | Glucose | 635 | 3 | 43 | 41 | 9 | 3.6 |
| 75 | Glucose + aa | 454 | 2 | 33 | 32 | 22 | 10 |

foci of PolC-GFP (Fig. 1C), whereas after about two generations in the absence of IPTG (decreased expression of *dnaA*), ~80% of the cells lacked the visible foci of PolC-GFP (Fig. 1D). Thus, the foci depend on continued DNA replication and are not simply storage repositories for inactive fusion proteins.

To sustain growth rates that are faster than the time it takes to replicate the chromosome, bacteria undergo multifork replication; from *oriC*, new rounds of DNA replication begin before the previous round of replication has finished. Thus, at faster growth rates, there are multiple DNA replication forks in a single cell (*3, 8*). We found that the number of PolC-GFP foci per cell increased at faster growth rates (Table 1). Together, these results indicate that the visible foci of PolC-GFP are most likely associated with active DNA replication forks.

A single fork has at least two PolC molecules, one each on the leading and lagging strands. It is not clear whether two molecules of PolC-GFP would be detectable, but there are ~40 molecules of PolC per cell (*4*); we postulate that most of these PolC molecules are at or near the replication fork and are perhaps involved in mismatch repair of newly replicated DNA.

The simplest pattern of PolC-GFP localization was observed in cells growing slowly with succinate as the carbon source. Approximately 25% of the cells did not have visible foci of PolC-GFP (Table 1), but they had a very faint diffuse green fluorescence throughout the cell. These cells were probably not replicating DNA [consistent with the expectation that, during very slow growth, there is a gap between periods of DNA synthesis (*9*), which is analogous to the gap periods in eukaryotes]. The majority of cells (~56%) had a single focus of PolC-GFP (Table 1) at or near midcell (Figs. 1E and 2A). Approximately 19% of the cells had two foci of PolC-GFP (Figs. 1, F and G, and 2B): ~15% with foci that were close together (≤35% of the cell length apart) and positioned near midcell (Figs. 1F and 2C) and ~5% with foci near the quarter positions (Figs. 1G and 2D), which become midcell after division. The ~5% were probably cells in which reinitiation of replication occurred before cell division and were not cells in which the two foci near midcell moved to the quarter positions. This interpretation was supported by the observation that, at faster growth rates, some cells have four foci (two close together at midcell and one at each quarter position).

Cells undergoing replication have at least two replication forks: one synthesizing clockwise on the circular chromosome and the other synthesizing counterclockwise. Our results indicated that, for ~80% of the replication cycle, the two forks were close together and could not be resolved (*10*) but that, during the last ~20% of the replication cycle, the forks were far enough apart so that we saw two discrete foci that were positioned around midcell (Figs. 1F and 2C). The separation of the forks could be regulated, or it could simply be due to cell growth. Presumably, at the termination of replication, one fork releases the template first so the DNA is not stretched between the two forks. The simplest interpretation of our results is that the DNA replication machinery is positioned primarily at or near midcell. A diagram of the replication and division cycles for cells growing slowly is shown in Fig. 3A.

The pattern of PolC-GFP localization was more complex at faster growth rates because of multifork replication (Table 1). With glucose as the carbon source, only ~3% of the cells lacked visible foci, and a substantial fraction (~12%) had three or four foci; this observation indicates that, as expected, the gaps between replication cycles, which were observed during slow growth, are gone and new rounds of replication begin before previous rounds finish. The position of PolC-GFP in cells with one or two foci was similar to that described for cells growing slowly [a single focus at or near midcell (Fig. 2E), two foci near midcell (Fig. 2F), and foci near each quarter position (Fig. 2G)]. In cells with three foci, PolC-GFP was near midcell and the quarters (Figs. 1H and 2H), most likely because of reinitiation from *oriC* at the cell quarters before the forks from the first round separated at midcell. A small percentage of cells had four foci of PolC-GFP (Table 1) [two foci near midcell and one near each of the cell quarters (Fig. 1I)]. The foci near midcell were probably the two old forks that had separated, and the foci at the quarters were probably the two new sets of replication forks that resulted from reinitiation at *oriC*. The existence of cells with three and four foci indicates that the foci at the quarter positions

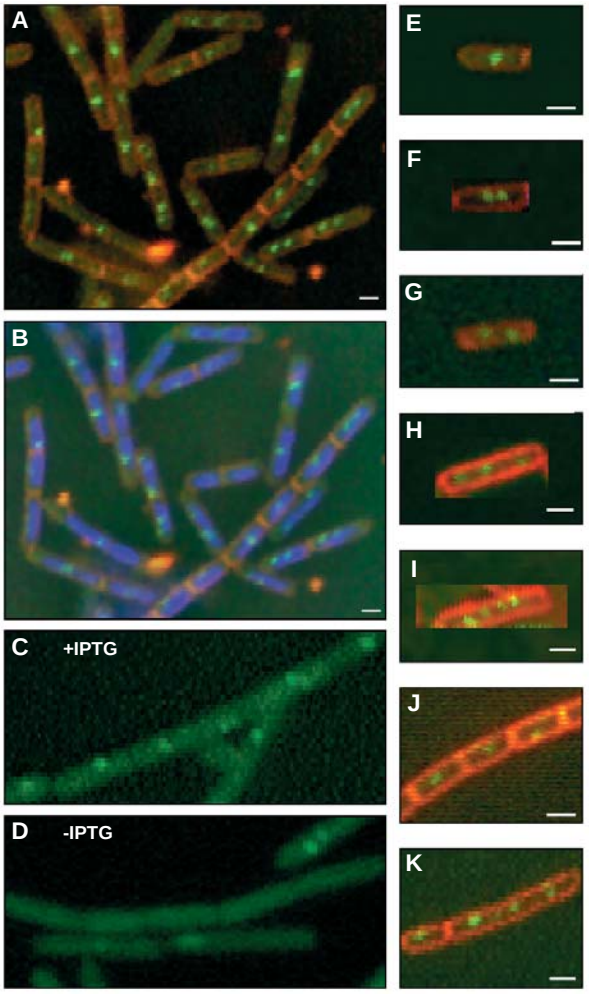


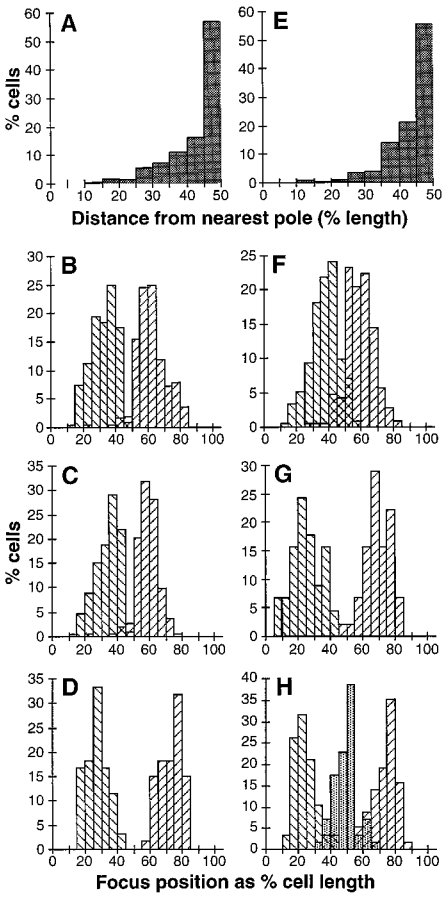

**Fig. 1.** Localization of replicative DNA polymerase in living cells. (**A** and **B**) PolC-GFP localized in discrete foci. Cell outlines were visualized (orange) with the vital membrane stain FM4-64 (Molecular Probes; Eugene, OR) simultaneously with the GFP signal (green). DNA was stained with DAPI, and images were superimposed with those from (A) to generate (B). Images were captured with a cooled CCD camera (*6*). Cells containing *polC-gfp* (strain KPL304) were grown to midexponential phase at 30°C in defined minimal medium (*21*) containing glucose (1%), glutamate (0.1%), required amino acids (40 μg/ml), and spectinomycin (40 μg/ml) to maintain the selection for the *polC-gfp* fusion (doubling time, ~115 min). A similar localization of PolC-GFP at or near midcell was observed by IFM in fixed cells with antibodies to GFP (*22*). (**C** and **D**) Visualization of PolC-GFP is dependent on DNA synthesis. Cells containing *dnaA* fused to Pspac were grown in defined minimal glucose medium in the presence or absence of IPTG (*7*). PolC-GFP was visualized as in (A). After about two generations (~4 hours) in the absence of IPTG, few cells contained PolC-GFP foci. (**E** through **G**) Cells were grown as in (A) except that sodium succinate (1%) replaced glucose as the carbon source (doubling time, ~230 min). (E) A cell with a single focus of PolC-GFP localized at or near midcell. (F) A cell with two foci of PolC-GFP together near midcell. (G) A cell with a focus of PolC-GFP near each cell quarter. (**H** and **I**) Cells were grown with glucose (1%) as described in (A) (doubling time, ~115 min). (H) A cell with three foci of PolC-GFP (one near midcell and one near each cell quarter). (I) A cell with four foci of PolC-GFP (two together near midcell and one near each quarter position). (**J** and **K**) Localization of τ-GFP (J) and δ′-GFP (K). Cells were grown as described in (A). Scale bars, 1 μm.

probably resulted from new initiation events and not from a migration of the foci from midcell (Fig. 3B). We fused GFP to two other proteins that were present at the replication fork (*11*) and found that both τ-GFP and δ′-GFP fusions were functional and had patterns of localization that were similar to those of PolC-GFP (Fig. 1, J and K).

The basic components of the DNA replication complex are conserved (*4*), and it is likely that other organisms also have a stationary replisome. We postulate that the location of the *Escherichia coli* SeqA protein indicates the location of the *E. coli* replisome. SeqA binds hemimethylated DNA (*12*) and localizes in discrete foci (*13*) in a pattern very similar to that of *B. subtilis* DNA polymerase. The highest concentration of hemimethylated DNA should be near the replication fork because of the lag between replication and remethylation (*14*). The dependence of SeqA localization on the Dam methylase (*13*) is consistent with this model. [*B. subtilis* does not have a *seqA* homolog (*2*)].

Our results show that the replicative DNA polymerase of *B. subtilis* is found at discrete intracellular positions (predominantly midcell) and is not randomly distributed along the nucleoid mass. If the replisome is anchored in place, then the overwhelming implication is that the DNA template moves. This is consistent with the factory model in which the DNA polymerase pulls the DNA through itself during replication. We speculate that DNA polymerase is recruited to midcell by replication initiation proteins.

The position of the replicative DNA polymerase fits with the emerging model for bacterial chromosome duplication and segregation. Newly replicated *oriC* regions are actively separated from each other and then maintained near opposite cell poles for most of the cell cycle (*15–17*). We propose that the extrusion of the newly replicated DNA from a stationary polymerase at midcell may be sufficient to cause the separation of sister origins, perhaps in the absence of additional postulated mitotic-like spindles and motors. With sister *oriC* regions maintained near opposite poles and DNA replication occurring at midcell, the bulk of the newly replicated chromosomes may be segregated from each other by a combination of extrusion from the polymerase and chromosome compaction and refolding (*17–19*).



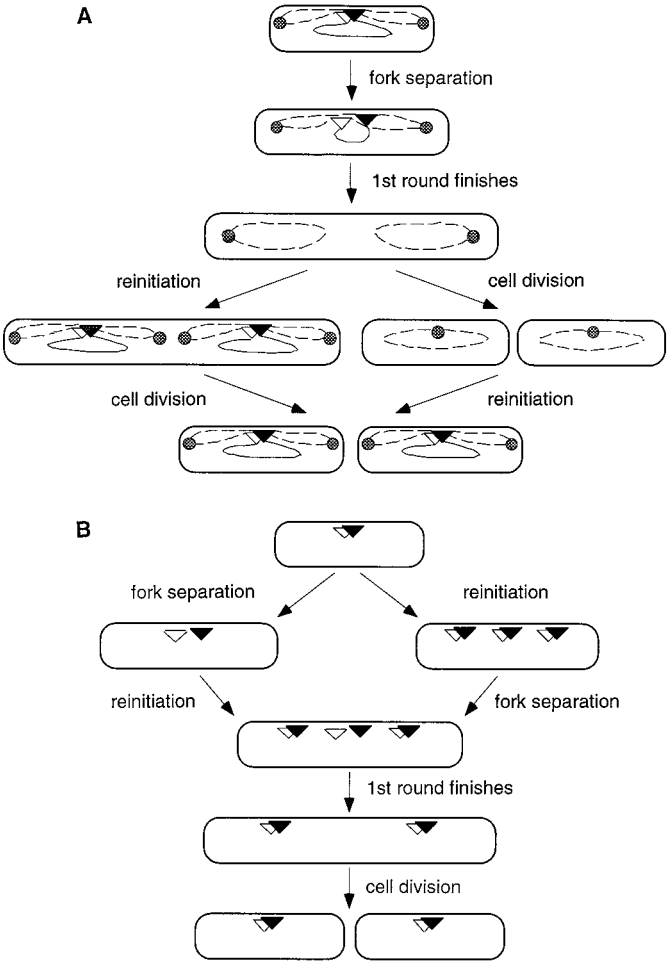




**Fig. 2 (left).** Foci of PolC-GFP localized to discrete intracellular positions. The positions of the approximate centers of PolC-GFP foci were measured in relation to a cell pole, and the positions were converted to the percentage of cell length. For all cells with a single focus [(A) and (E)], the distance to the closest pole was measured. For cells with multiple foci [(B) through (D) and (F) through (H)], distances were measured from a single pole that was chosen randomly. The two [(B) through (D), (F), and (G)] or three (H) different bar patterns (diagonal lines and dots) represent the different foci in a given cell. The number of cells with a focus at a given position was divided by the total number of cells with the indicated number of foci to give the percentage of cells for each graph. (**A** through **D**) Samples were taken from cells at midexponential growth in defined minimal medium with succinate as the carbon source (doubling time, ~230 min). (A) Of the 742 cells with a single focus of PolC-GFP, over 50% had a focus at midcell. (B) All cells (252) with two foci of PolC-GFP are represented. Cells with two foci were divided into two classes based on the distance between foci; 192 cells had foci ≤35% of the cell length apart (C), and 60 cells had foci ≥36% of the cell length apart (D). Foci at the quarter positions should be 50% of the cell length apart. The 35% of the cell length cutoff was chosen on the basis of the size of the foci (~10% of cell length) and the drift from midcell that was seen in cells with a single focus. (**E** through **H**) Samples were taken from cells at midexponential growth in defined minimal medium with glucose as the carbon source (doubling time, ~115 min). (E) Cells (274 total) with a single focus of PolC-GFP. (F and G) Cells with two foci were divided into two classes based on the distance between foci; 215 cells had foci ≤35% of the cell length apart (F), and 45 cells had foci ≥36% of the cell length apart (G). (H) Cells (57 total) with three foci of PolC-GFP are represented. **Fig. 3 (right).** A model for the localization of the replicative DNA polymerase in *B. subtilis*. Triangles represent single replication forks; overlapping triangles represent replication forks going in opposite directions that cannot be resolved by microscopy. (**A**) The location of DNA polymerase during slow growth. Dashed lines, newly replicated chromosomes; solid lines, template chromosomes. The shaded circles represent the origin regions. (**B**) The location of DNA polymerase during multifork replication in rapidly growing cells.

**References and Notes**

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6. The GFP fusion proteins were visualized in living cells as in (*16*) and (*20*). About 2 μl of agarose (0.5 to 1%, in medium) was applied to a microscope slide, allowed to cool for ~30 s, and then ~10 μl of culture was added. After 2 to 5 min, excess liquid was aspirated, and a glass coverslip was placed on the slide. Cell membranes were stained by growing the cells for at least one doubling in the red membrane dye FM4-64 (50 to 70 ng/ml) (Molecular Probes, Eugene, OR). The cell outlines were visualized simultaneously with the GFP signal using Chroma filter set no. 41012. Microscopy was performed with a Zeiss Axioplan II. Images were captured with a cooled charge-coupled device (CCD) camera (Optronics Engineering, Goleta, CA) and a CG-7 frame grabber (Scion, Frederick, MD) with Scion Image 1.62 software.
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10. This estimate is based on the view that cells with a single focus (742 cells) and cells with two foci close together (192 cells) represented DNA initiation events that occurred at midcell and were part of the same replication cycle. Thus, the two replication forks are resolved for ~20% (192 out of 934 cells) of the replication cycle.
11. Strains KPL382 and KPL386 contain *dnaX-gfp* (τ-GFP) and *holB-gfp* (δ′-GFP) fusions, respectively, that were integrated into the chromosome and were under control of the endogenous promoters. The constructs were verified by PCR analysis of chromosomal DNA. τ dimerizes the leading and lagging strand polymerases, and δ′ is one of the proteins involved in the assembly of the sliding clamp that is responsible for processivity of replication (*2*–*4*). The fusions were constructed in a manner similar to that for *polC-gfp* (*5*), except that the GFP variant GFPmut2 (mutations $Ser^{65} \rightarrow Thr^{65}$, $Val^{68} \rightarrow Leu^{68}$, and $Ser^{72} \rightarrow Ala^{72}$) [B. P. Cormack, R. H. Valdivia, S. Falkow, *Gene* **173**, 33 (1996)] was used. *gfpmut2* was amplified by PCR so that the product had a 5′ in-frame Xho I site before the first codon and an Sph I site downstream of the stop codon. pKL147 and pKL148 contain, respectively, the 3′ end of *dnaX* (τ) and the 3′ end of *holB* (δ′), fused in-frame to *gfpmut2*, with a five–amino acid linker (Leu-Glu-Gly-Ser-Gly), and inserted into the integrative vector pUS19 [A. K. Benson and W. G. Haldenwang, *J. Bacteriol.* **175**, 2347 (1993)]. The 3′ ends of *dnaX* and *holB* were amplified by PCR so that the stop codons were changed to Xho I restriction sites. Sequences of all primers are available upon request.
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23. We thank S. Sanders, P. Levin, K. Pogliano, and J. Roberts for advice; B. Cormack for *gfpmut2*; R. Losick, A.L. Sonenshein, and S. Bell for comments on the manuscript; and members of our lab for discussions and comments on the manuscript. Supported in part by PHS grant GM41934 (A.D.G.).

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