Cell, Vol. 88, 667–674, March 7, 1997, Copyright ©1997 by Cell Press

Bipolar Localization of the Replication Origin Regions of Chromosomes in Vegetative and Sporulating Cells of B. subtilis

Chris D. Webb,*# Aurelio Teleman,*# Scott Gordon,† Aaron Straight,‡ Andrew Belmont,§ Daniel Chi-Hong Lin,‖ Alan D. Grossman,‖ Andrew Wright,† and Richard Losick*
*Department of Molecular and Cellular Biology
Harvard University
Cambridge, Massachusetts 02138
†Department of Molecular Biology and Microbiology
Tufts University Health Sciences Campus
Boston, Massachusetts 02111
‡Department of Physiology
School of Medicine
University of California
San Francisco, California 94143
§Department of Cell and Structural Biology
University of Illinois, Urbana–Champaign
Urbana, Illinois 61801
‖Department of Biology
Massachusetts Institute of Technology
Cambridge, Massachusetts 02139

Summary

To investigate chromosome segregation in B. subtilis, we introduced tandem copies of the lactose operon operator into the chromosome near the replication origin or terminus. We then visualized the position of the operator cassettes with green fluorescent protein fused to the LacI repressor. In sporulating bacteria, which undergo asymmetric cell division, origins localized near each pole of the cell whereas termini were restricted to the middle. In growing cells, which undergo binary fission, origins were observed at various positions but preferentially toward the poles early in the cell cycle. In contrast, termini showed little preference for the poles. These results indicate the existence of a mitotic-like apparatus that is responsible for moving the origin regions of newly formed chromosomes toward opposite ends of the cell.

Introduction

The segregation of homologous chromosomes during the mitotic cycle of eukaryotic cells is mediated by a microtubule-based spindle that pulls chromosomes to opposite poles of the cell prior to cytokinesis (Hyman, 1995; Pluta et al., 1995; Barton and Goldstein, 1996). Bacteria, in contrast, lack a conspicuous apparatus for chromosome segregation, and the nature of the machinery for ensuring that newly duplicated chromosomes are faithfully distributed to daughter cells is obscure for prokaryotes (Wake and Errington, 1995). In formulating their replicon model for the regulation of DNA replication, Jacob et al. (1963) called attention to the DNA segregation problem in bacteria, envisioning that the attachment of newly duplicated chromosomal origins to the cell surface and growth between the points of attachment could progressively pull the two chromosomes apart. Lineage studies have demonstrated that DNA segregates in a nonrandom fashion (Eberle and Lark, 1966; Cooper, 1991), and evidence has been obtained for the association of the replication origin region with the cytoplasmic membrane (Bone et al., 1985; Sandler and Keynan, 1988). Nonetheless, zonal growth is not a general feature of cell wall growth, and it is unlikely that this is the basis for the distribution of newly formed chromosomes to daughter cells (Mobley et al., 1984; Woldringh et al., 1987).

Some progress has been made in addressing the chromosome segregation problem in bacteria through the identification of partition (*par*) genes, some of which govern the segregation of plasmid replicons whereas others influence the efficiency of chromosome segregation (Hiraga, 1992). Nonetheless, the problem of chromosome movement has resisted analysis in part because of the small size of bacteria and the absence of compact morphological structures equivalent to the highly condensed chromatids of eukaryotic cells that can be visualized by light microscopy.

To address the issue of chromosome movement in bacteria, we took advantage of a method recently devised by Straight et al. (1996) and Robinett et al. (1996) for visualizing specific chromosomal sites during mitosis in budding yeast and in Chinese Hamster ovary cells. The method is based on the insertion into the chromosome of a cassette consisting of multiple (256) tandem copies of the operator for the E. coli lactose operon (*lacO*) and the use of a fusion of the green fluorescent protein (GFP) from Aequorea victoria to the lactose operon repressor LacI. Binding of GFP-LacI to the tandem operators causes the hybrid repressor molecules to cluster in a small spot that can be visualized by fluorescence microscopy.

Our strategy for visualizing chromosome segregation in bacteria was to insert a copy of the *lacO* cassette either near the replication origin or near the terminus of the chromosome of Bacillus subtilis. This spore-forming bacterium was advantageous for use in these studies because previous genetic experiments had led to the inference that at the start of the process of spore formation, when the sporangium contains two newly formed chromosomes, the replication origins become attached near opposite poles of the cell (Wu and Errington, 1994). We asked whether we could directly visualize polar attachment of the replication origins at the start of sporulation. We also asked whether polar attachment was a normal feature of the cell cycle during binary fission. Here we report that the origins, but not the termini, of the two sporangial chromosomes do indeed localize near the ends of the sporangium. Moreover, we detect a population of cells at an early stage of the cell cycle in which the replication origin is preferentially located near the cell poles, a finding that indicates the existence of a mitotic-like apparatus that is responsible for moving the replication origin regions of newly duplicated chromosomes toward opposite ends of the cell.

# These authors contributed equally to this work.

Results and Discussion

Bipolar Localization of the Replication Origins during Sporulation

We first sought to visualize the replication origin and terminus in cells undergoing sporulation because the results of previous genetic studies had suggested that the region of the chromosome proximal to the origin is sequestered near the cell pole (Wu and Errington, 1994). Sporulating cells undergo a modified form of cell division in which a septum is formed at an extreme polar position to create a small forespore cell and a large mother cell. Unlike the process of binary fission in which DNA segregation precedes cytokinesis, the sporulation septum is formed prior to translocation of a complete chromosome into the forespore. Initially after polar septation, only a small portion of the chromosome is trapped in the forespore; the remainder is subsequently transported across the septum into the small sporangial compartment by a conjugation-like protein called SpoIIIE (Wu et al., 1995). Mutants defective in the *spoIIIE* gene are blocked in DNA translocation and hence their forespore compartment contains only about one-third of the normal complement of DNA (Wu and Errington, 1994; Sharpe and Errington, 1995). That this one-third corresponds to the origin-proximal region of the chromosome is inferred from the following chromosomal position effect on the expression of genes under the control of the forespore-specific transcription factor σ^F: in sporulating cells of a *spoIIIE* mutant, σ^F-controlled genes located proximal to the replication origin are actively transcribed whereas genes located distal to the origin are transcriptionally silent (Sun et al., 1991). This has been interpreted (Wu and Errington, 1994) to indicate that the replication origins are attached near the cell poles at the start of sporulation and that, in the absence of DNA translocation, only origin-proximal DNA is present in the compartment in which σ^F is active.

To observe the origin and the terminus in sporulating cells, we engineered the synthesis of the GFP-LacI fusion protein in sporulating cells using a promoter (*spoVG*) that is active at the onset of sporulation (Zuber and Losick, 1983). The GFP-LacI construct was then introduced into cells that contained the operator cassette located near the replication origin region of the chromosome (at *amyE* [25°] or *spo0J* [359°]) or near the terminus (*cgeD* [181°]).

GFP-LacI-producing bacteria that harbored the *lacO* cassette near the replication origin characteristically exhibited two green fluorescent dots near opposite poles of the cell (Figures 1A and 1E). Due to the low level of the fluorescent signal and a moderate level (30%–40%) of sporulation in our engineered strain, only a minority of the cells exhibited green dots (~11%). In other work involving the use of a cooled charge coupled device (CCD) camera, a higher proportion of sporangia exhibiting fluorescence was observed (~30%). Of those sporangia in which green dots were observed, a substantial proportion (61%, out of a total of 95 cells examined) exhibited a bipolar pattern of fluorescence (in many of the remaining sporangia only a single polarly localized dot was seen). When the *gfp-lacI* fusion was introduced into bacteria lacking the operator cassette, no green dots were observed above a uniform background of low level fluorescence.

Bacteria exhibiting green dots were mostly observed at an early stage of development, prior to the formation of the polar septum (Figure 1B). Fluorescent sporangia were harder to detect at later stages of development, although some postseptation sporangia were observed that exhibited a bipolar pattern of fluorescence (Figure 2B). Yet later, at the stage at which the forespore is wholly engulfed by the mother cell, the origins no longer appeared to be attached near the poles of either compartment (Figure 2C).

In contrast to the results obtained with the operator cassette near the origin, GFP-LacI-producing bacteria harboring the *lacO* cassette near the terminus of replication exhibited one or two green dots that were characteristically located approximately in the middle of the cell (Figure 1C and 1G). For sporangia in which only one dot was evident, the two chromosomes may not have fully completed replication. Alternatively, these sporangia may contain two fully duplicated chromosomes but the termini may be in such close proximity that they could not be resolved by fluorescence microscopy. Once again, fluorescence was principally observed in sporangia at an early stage of development (Figure 1D). Among cells with a signal (11% of the total), a high proportion (74% out of a total of 149 cells) exhibited green dots in the middle region of the cells, and rarely, if ever, near both poles.

In summary, our observations are consistent with the idea depicted in Figure 3A that at the start of sporulation, chromosomes are oriented with their origins near opposite poles and their termini in close proximity to each other near the middle of the cell (i). Next, after polar septation (ii), the origin-distal region of the forespore chromosome is translocated into the small sporangial compartment (iii). Finally, after engulfment of the forespore by the mother cell, the replication origin ceases to be attached near either end of the sporangium (iv).

Polyploidy in a Partition Mutant

Recent genetic experiments have implicated the *spo0J* gene in the anchoring of the replication origin region to the sporangial poles (Sharpe and Errington, 1996). Mutation of *spo0J*, which is related to the family of partition genes that are required for proper plasmid segregation in Gram-negative bacteria (Hiraga, 1992), causes a defect in chromosome segregation during growth and a block at the onset of sporulation (Ireton et al., 1994). This sporulation block can be overcome by a second mutation in *soj* (for *suppressor of spo0J*), which is also related to partition genes. Thus, a *soj spo0J* double mutant is capable of sporulating (Ireton et al., 1994). Interestingly, however, the strict chromosome position effect observed in a *spoIIIE* DNA translocase mutant is not observed in the presence of additional mutations in *soj* and *spo0J*. Thus, in a *soj spo0J spoIIIE* triple mutant, a low level of expression of σ^F-controlled genes located distal to the replication origin can be observed (Sharpe and Errington, 1996). These observations have been interpreted to indicate that *spo0J* is involved in polar attachment of the replication origins and that, in its absence, the chromosomes are not oriented and random

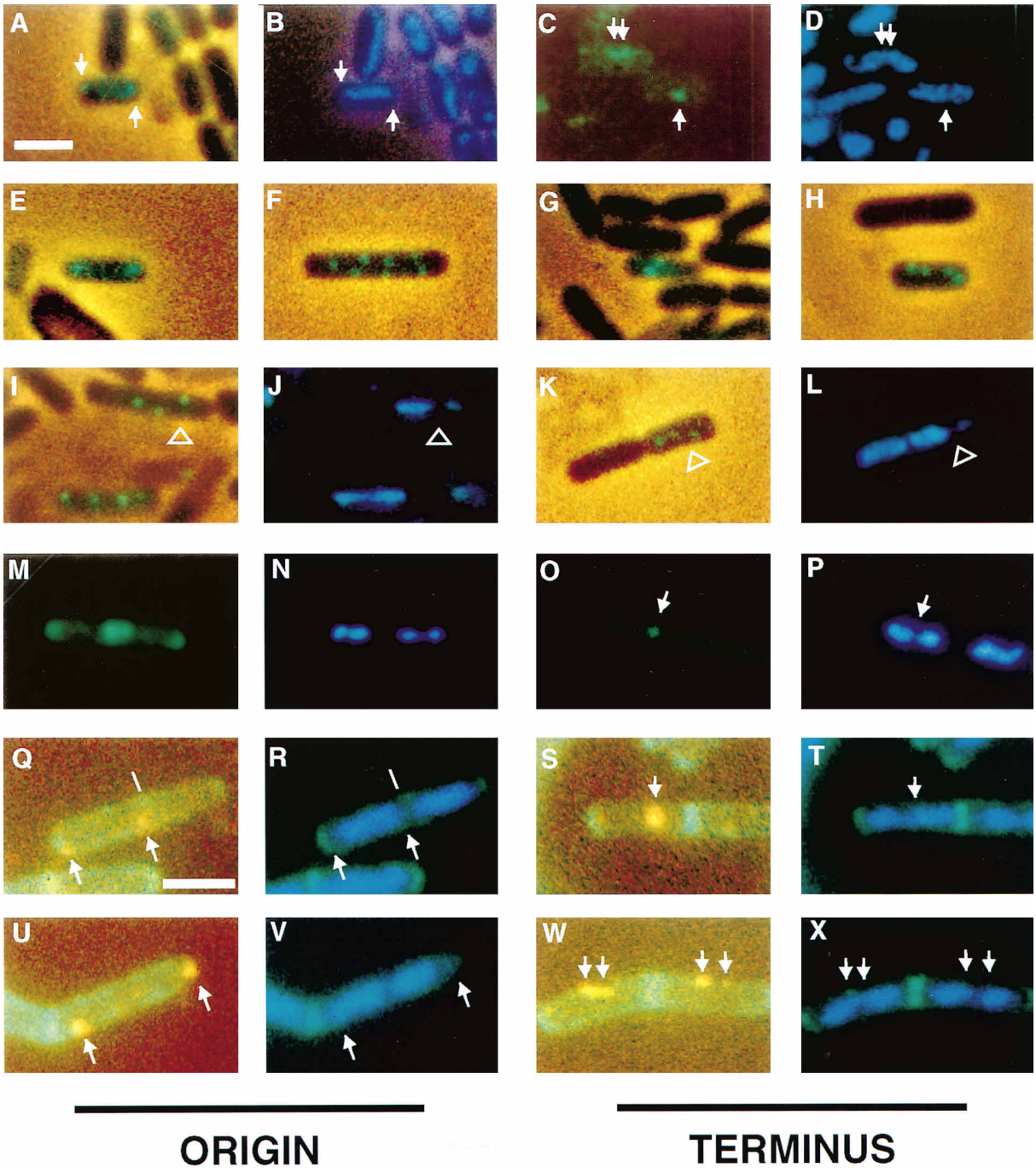

Figure 1. Fluorescence Micrographs of Sporulating and Vegetative Cells with the Operator Cassette Near the Origin or the Terminus

The operator cassette is near the origin (*amyE*) in (A), (B), (E), (F), (I), (J), (M), (N), (Q), (R), (U), and (V) and near the terminus (*cgeD*) in (C), (D), (G), (H), (K), (L), (O), (P), (S), (T), (W), and (X). Arrows indicate the location of dots from GFP-LacI. (A, B, and E) Sporangia (strain AT16) exhibiting bipolar dots from the origin region (green in [A] and [E]) and a predivisional pattern of unsegregated chromosomal DNA (blue in [B], corresponding to sporangium in [A]). (C, D and G) Sporangia (strain AT18) with one or two dots from the terminus region and a predivisional pattern of chromosomal DNA ([C] and [D] show the same field of cells). (F, H–L) Multiple dots in *soj spo0J* mutant sporangia, with the operator cassette near the origin (F, I–J; strain AT19) or near the terminus (H, K–L; strain AT20). Note the gap between the forespore and mother cell chromosome as indicated by open arrowheads in (J) and (L). (M) Bipolar pattern of dots from the origin region in a pair of vegetative cells (strain CW437), with separated nucleoids in each (N). (O) Single dot from the terminus region in the middle of a vegetative cell (strain CW438), with nucleoids in the process of separating, seen in (P). (Q–X) Immunofluorescence of vegetative cells stained with anti-GFP antibodies (orange), with lectin to stain the cell wall (green), and with DAPI to visualize DNA (blue). (Q and R) Bipolar pattern of dots from the origin region (strain CW437) seen early in the cell cycle, prior to chromosome segregation. Line indicates location of a lectin-stained septum, not clearly seen in photograph. (U and V) Bipolar pattern of origin region dots (strain CW437) later in the cell cycle when the chromosomes have separated into two nucleoids. (S and T) Single dot from the terminus in a cell (strain CW438) in which the nucleoids have not yet fully separated. (W and X) Two dots from the terminus (strain CW438) seen in adjacent cells in which the nucleoids have begun to separate (left-hand cell) or have fully separated (right-hand cell). The scale bar in (A), which represents 2 μm, pertains to (A)–(P), whereas the scale bar in (Q), which also represents 2 μm, pertains to (Q)–(X).

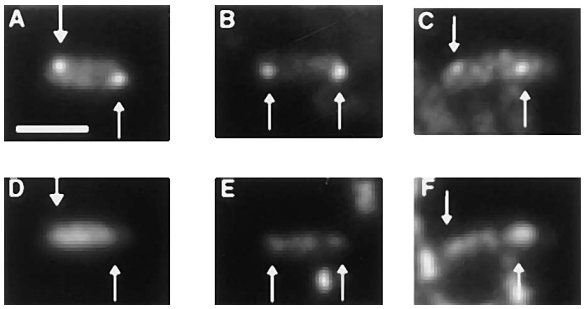

Figure 2. Fluorescence Micrographs of Sporangia with the Operator Cassette Inserted Near the Origin

Strain AT25 (with the operator cassette at 359°) is shown. The upper panels show fluorescence from the GFP-LacI fusion in a predivisional sporangium (A), in a postseptation sporangium (B) and in a postengulfment sporangium (C). The corresponding lower panels (D–F) show fluorescence from DNA stained with DAPI for each of the same sporangia. Arrows indicate the locations of dots from GFP-LacI.

regions of the chromosome become trapped in the forespore when the polar septum is formed. Indeed, consistent with the idea that Spo0J could be involved in sequestering the replication origin near the poles, other work (D. C.-H. L., P. Levin, A. G., unpublished data) has shown that Spo0J exhibits a bipolar distribution during sporulation (as well as during growth; see below).

To investigate the role of these partition genes in the polar attachment of the origin region during sporulation, we introduced the *gfp-lacI* construct and the *lac* operator cassette into a *soj spo0J* double mutant. Strikingly, multiple dots were observed when the operator cassette was located either near the origin or near the terminus (Figures 1F, 1H, 1I, and 1K). Among sporangia exhibiting a fluorescent signal, 53% (out of a total of 156 cells) had more than two dots. Moreover, sporangia were observed with up to eight dots when the cassette was located near the origin and up to four dots when the cassette was at the terminus. Because multiple origins and multiple termini were observed, some of the *soj spo0J* mutant sporangia must contain more than two complete copies of the chromosome. This is also evident from the size of the mutant cells: those cells with multiple dots were significantly larger than other cells (for example, compare the sporangium in Figure 1F with that in Figure 1E).

Most of the mutant sporangia exhibiting a fluorescent signal were at the predivisional stage of sporulation, but some sporangia with multiple chromosomes were observed that had appeared to reach the stage of polar division. These could be recognized by means of DAPI staining, which revealed a separated region of DNA, indicative of the presence of a forespore (Setlow et al., 1991; Resnekov et al., 1996) (Figures 1J and 1L). The existence of postseptation sporangia with multiple chromosomes suggests a possible alternative explanation for the absence of a strict chromosomal position effect in a *soj spo0J spoIIIE* triple mutant: polar septation in a sporangium containing multiple chromosomes could result in the trapping of random chromosomal regions within the newly formed forespore due to the presence of extra copies of the chromosome in the sporangium

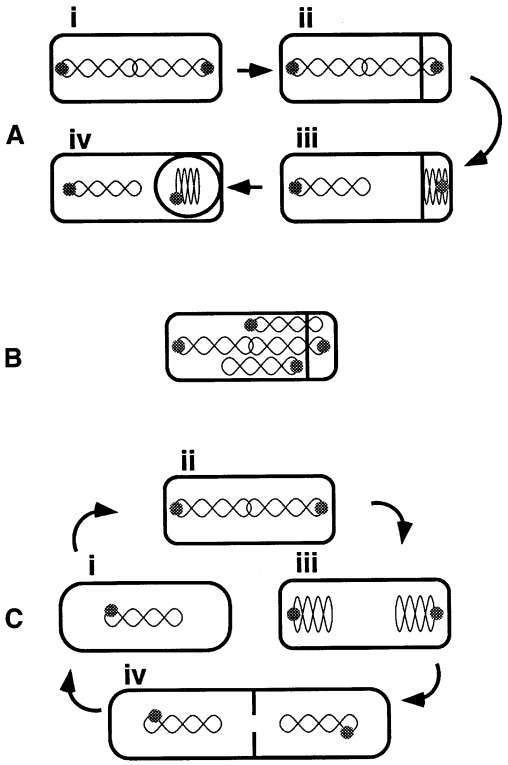

Figure 3. Model Depicting the Position of the Origin during Asymmetric and Symmetric Cell Division

Dots indicate the location of the origin on the chromosome.

(A) Movement of the origin during sporulation. (i) Prior to septation the two chromosomes appear as a single elongated filament and the origins are localized near opposite poles. (ii) Initially after polar septation, the origin proximal region of the forespore chromosome is trapped in the forespore. (iii) After DNA translocation, an entire chromosome is packed into the forespore. (iv) After engulfment of the forespore by the mother cell, the origins are detached from the poles.

(B) Multiple chromosomes in a *soj spo0J* double mutant sporangium. The presence of multiple chromosomes could allow the origin distal regions of an extra chromosome to become trapped in the forespore.

(C) Movement of the origin during binary fission. (i) Early in the cell cycle a single unreplicated chromosome is spread across the cells as an elongated filament. (ii) Next, following replication, the origins localize to opposite poles. (iii) Later, the chromosomes condense, creating a gap in which the septum can form. (iv) Cytokinesis.

(Figure 3B). According to this view, Spo0J need not be responsible for anchoring the origin region to the poles. Instead, it may serve to coordinate cell division with chromosome copy number in cells entering sporulation so that normally only two chromosomes are present in cells undergoing polar division. Alternatively, Spo0J could be involved both in chromosome anchoring as well as in coordinating chromosome copy number with cell division.

Polar Localization of the Origin during Vegetative Growth

Next, we asked whether polar attachment of the replication origin region was unique to cells entering sporulation or a general feature of chromosome segregation

during asymmetric and symmetric cell division. To visualize the origin and terminus in growing cells, we engineered the expression of *gfp-lacI* in vegetative cells by placing the gene (*spo0H*) for σ^H under the control of the xylose-inducible promoter (Dubnau et al., 1988; Gartner et al., 1988). Thus, in such cells, xylose would enhance the levels of σ^H during growth and, hence, stimulate transcription of *gfp-lacI* from the σ^H-controlled *spoVG* promoter. Nevertheless, only a low level of green fluorescence was observed in such engineered cells.

To increase the sensitivity at which GFP-LacI could be detected in growing cells, we turned to immunofluorescence using antibodies directed against GFP, an effective strategy that enabled us to observe signals for the GFP-LacI fusion in over 40% of the cells. In the following experiments, GFP-LacI appeared as orange dots whereas the cell wall and septa were stained green by the use of a lectin. Finally, nucleoids were visualized by the use of DAPI. The use of DAPI enabled us to distinguish cells at early stages of the cell cycle in which the nucleoid appears as a single filament along the length of the cell (Figures 1R and 1T) from cells at late stages of the cycle when the chromosomes have segregated into two distinct condensed nucleoids (Figures 1V and 1X).

First, we consider cells in exponential growth phase harboring the operator cassette near the origin. Among cells for which a signal could be seen, most (75%, out of a total of 140 cells) exhibited only a single dot, which was often located near a cell pole. The significance of such one-dot cells was difficult to interpret. They could represent cells in which the origin had not replicated or in which newly formed origins had not yet begun to segregate from each other. Alternatively, such cells could have contained two well-separated origins but, because of the low level of signal detection during growth, a second origin located out of the plane of focus might not have been detected. More informative was the case of cells exhibiting two dots (25%, out of a total of 140 cells). Among such cases, cells were often observed to have a dot close to each end. This bipolar pattern of dots was most prevalent at early stages of the cell cycle, when the chromosomes had not yet segregated (Figures 1Q and 1R), but could also be observed at later stages when the chromosomes had separated into two distinct nucleoids (Figures 1M, 1N, 1U, and 1V). A similar pattern of bipolar localization was observed in experiments designed to visualize Spo0J in growing cells, reinforcing the view that Spo0J binds to the replication origin region and providing independent evidence that chromosomes become oriented with their origins toward the poles (D. C.-H. L. et al., unpublished data). Recently, the partition homologs ParA and ParB of the gram-negative bacterium Caulobacter crescentus have also been found to exhibit a bipolar pattern of localization, an indication that the orientation of the chromosomes with the origin toward the poles of the cell could be a general feature of chromosome segregation in prokaryotes (Mohl and Gober, 1997).

Next, we consider growing cells harboring the operator cassette near the terminus. Such cells generally displayed one or two dots located approximately in the middle of the cell (Figures 1O, 1S, and 1W). Although cells exhibiting two dots were infrequent, a bipolar localization pattern of termini tagged with the operator cassette was rarely if ever observed. Thus, our results indicate the existence of a population of growing cells in which newly formed origins, but not termini, are sequestered at or near opposite ends of the cell.

The above experiments were carried out with cells that were grown in a rich medium, conditions of rapid growth in which multiple replication forks are to be expected. When the generation time is shorter than the time required for duplication of a chromosome, a second round of replication is initiated before the first round of replication is complete (Cooper, 1991). Despite this expectation, we were not able to observe more than two dots in cells in which the origin was tagged with the *lacO* cassette. Thus, if multiple replication forks were present in rich medium, we were not able to detect them under our conditions. However, using a newly constructed strain (see below) that exhibits brighter fluorescence, we do observe multiple dots in rapidly growing cells. In light of this issue, we also carried out experiments with cells grown in a minimal medium (with glucose as the sole carbon source) in which no more than two replication forks were expected to be present. In minimal-medium grown cells with the *lacO* cassette at the origin, we detected cells with two dots (data not shown). Among such cells were those in which the dots were located in a bipolar fashion, similar to that observed for cells grown in rich medium.

Finally, to improve the efficiency with which operator-bound GFP-LacI could be detected in growing cells, we created an additional fusion in which *gfp-lacI* was joined to a strong vegetative promoter (*veg*; Moran et al., 1982). Minimal-medium grown cells having the P_{veg}-*gfp-lacI* fusion exhibited much brighter signals than in the experiments described above (data not shown). Moreover, a high proportion ($>$90%) of the cells exhibited detectable fluorescence. As before, among cells harboring the P_{veg}-*gfp-lacI* fusion, a subpopulation could readily be observed that exhibited a bipolar pattern of dots when the operator cassette was at the origin but not when it was at the terminus (data not shown).

A Model for Chromosome Segregation

Our results suggest the following model for chromosome segregation during binary fission. As depicted in Figure 3Ci, the newborn cell initially contains a single chromosome and hence a single replication origin region. Next, after replication commences, the two newly formed origins are drawn toward opposite ends of the cell, eventually resulting in a cell (Figure 3Cii) with two complete chromosomes, each anchored near a cell pole from a site (centromere) located in the vicinity of its replication origin. At this stage, the two chromosomes are spread across the length of the cell in the form of a single filament, similar to that observed early in sporulation. Cells (Figure 3Cii) with a bipolar arrangement of replication origin regions could arise in one of two ways. One possibility is that before the initiation of replication, the origin region is not attached near either pole. Only after replication commences do the origins migrate toward the poles. Alternatively, the origin region

in a newborn cell could be attached near a pole prior to replication. Then after replication ensues, one of the two newly formed origin regions could be transported toward the opposite end of the cell. Our data do not allow us to distinguish between these possibilities decisively.

In the next stage of the cell cycle (Figure 3Ciii), the two chromosomes separate from each other, moving to opposite ends of the cells. A simple way in which this could occur would be by condensation of the chromosomes, each anchored near an opposite end of the cell. This condensation could be mediated by a protein similar to members of the SMC family of eukaryotic chromosome condensation proteins, homologs of which are known to exist in bacteria (Hirano et al., 1995). Finally, after each chromosome has coalesced into a distinct nucleoid, cytokinesis (Figure 3Civ) occurs by the formation of a septum in the gap between the separated chromosomes. If these ideas are correct, then a principal challenge for future investigations will be to define the nature of the centromere and the mitotic-like apparatus that effects centromere movement and chromosome segregation.

Experimental Procedures

Strain Constructions

To express the *gfp-lacI* fusion of Straight et al. (1996) in B. subtilis, the fusion was placed under the control of the *spoVG* promoter by being joined in-frame at the third codon of the *spoVG* open reading frame (ORF). DNA from the *spoVG* promoter region was obtained by PCR amplification using oligonucleotides SDMOL1 (5′-GCTGGC GAAAGGGGGATGTG-3′), which corresponds to a vector sequence near the site of insertion of a *spoVG*-containing HindIII fragment in the HindIII site of pBSKS+, and ATO3 (5′-TCTAGGATCCCCATCGAT GTAACTTCCACAGTAGTTCACC-3′), which created adjacent ClaI and BamHI restriction sites after the third codon of the *spoVG* ORF. The PCR product was digested with EcoRI and BamHI and ligated to similarly digested pDG795 to create pAT10. Plasmid pDG795 is an MLS-resistance gene-containing vector for creating insertions at the ThrC locus on the B. subtilis chromosome (a gift of P. Stragier).

Next, the *gfp-lacI* gene fusion was amplified using pAFS78 (Straight et al., 1996) with oligonucleotides ATO1 (5′-AAAAAAGATC TGATTAAGTTGGGTAA-3′), which created a BglII site downstream of the open reading frame, and ATO4 (5′-ATAGCATCGATGAGTAAA GGAGA-3′), which created a ClaI restriction site 5′ of the ATG start codon. The resulting PCR product, digested with BglII and ClaI, was ligated to BamHI-ClaI-digested pAT10, yielding pAT11. This created an extended ORF in which three codons of *spoVG* were joined via a two codon linker to the *gfp-lacI* gene fusion. The plasmid was linearized by digestion with XhoI and used to transform wild-type, prototrophic B. subtilis strain PY79 (Youngman et al., 1984), followed by selection for MLS resistance (5 μg/ml). This yielded strain AT15, which was confirmed to be auxotrophic for threonine.

To insert the *lacO*-containing plasmid pAFS52 (Straight et al., 1996), into the chromosome near the origin (25°), we introduced into the chromosome at *amyE* a segment of DNA corresponding to the yeast *trp1* gene that was present in pAFS52. This 1.4 kb segment of pAFS52 was introduced into the *amyE* locus using the *amyE*-integrating vector pDG364 to create strain AT10. Next, a kanamycin resistance gene contained on a 1.9 kb BamHI-SalI fragment from pER82 (Driks et al., 1994) was introduced into pAFS52 to create pAT7. Strain AT10 was transformed with pAT7 followed by selection for kanamycin resistance (5 μg/ml). The resulting strain was then transformed with chromosomal DNA from AT15 followed by selection for MLS resistance (5 μg/ml), yielding AT16 which had both the *lacO* cassette at *amyE* (25°) and the *spoVG-gfp-lacI* fusion. To generate a *soj spo0J* mutant derivative of AT16, the strain was transformed with chromosomal DNA from AG1505 (Ireton et al., 1994) followed by selection for spectinomycin resistance (5 μg/ml), which yielded strain AT19.

To select for introduction of the *lacO* cassette in other regions of the chromosome of AT15, a chloramphenicol resistance gene (*cat*) contained on a 1.3 kb HindIII-SalI fragment from pDG364 (Cutting and Vander Horn, 1990) was introduced into a HindIII-SalI-digested pAFS52. This created pAT12, which was used to insert the *lacO* cassette at the sites near the origin (359°) and near the terminus (181°).

A 1 kb HindIII fragment from pIK217 corresponding to the *soj-spo0J* region of the chromosome (359°) (Ireton et al., 1994) was cloned into the HindIII site of pAT12, yielding pAT15. Finally, pAT15 was used to transform AT15 followed by selection for chloramphenicol resistance (5 μg/ml), generating strain, AT25.

To place the operator cassette near the terminus (181°), a 1.2 kb AatII-BglII fragment from pSR129 corresponding to the *cgeD* gene (Roels and Losick, 1995) was ligated to pAT12 that had been digested with AatII and BamHI, resulting in plasmid pAT14. This plasmid was used to transform AT15 followed by selection for chloramphenicol resistance (5 μg/ml), generating strain AT18. To generate a *soj spo0J* mutant derivative of AT18, the strain was transformed with chromosomal DNA from AG1505 (Ireton et al., 1994) followed by selection for spectinomycin resistance (100 μg/ml), which yielded strain AT20.

To express the *spoVG-gfp-lacI* fusion at higher levels during vegetative growth, an inducible *spo0H* fusion was made. A 550 bp SmaI-EcoRI fragment from pSG20H (Fort and Errington, 1985) containing part of *spo0H*, with ends rendered flush with DNA Polymerase I Klenow fragment, was cloned into a SalI-digested pDAG8–1, yielding pCW75. Plasmid pDAG8-1 has a xylose-inducible promoter-containing HindIII fragment from pDG1832 (a gift of P. Stragier) in the HindIII site of pJL74 (LeDeaux and Grossman, 1995). Plasmid pCW75 was inserted into the chromosome by a Campbell-like recombination at *spo0H* by transforming PY79 followed by selection for spectinomycin resistance (100 μg/ml), yielding CW436. Strains AT16, AT18, and AT25 were transformed with the chromosomal DNA of CW436 followed by selection for spectinomycin resistance (100 μg/ml) resulting in strains CW437, CW438, and AT29, respectively.

Growth Conditions

For vegetative growth, cells were grown overnight in Luria-Bertani (LB) medium at 25°C and then diluted 1:100 in either LB or S7 defined minimal medium (Jaacks et al., 1989) grown at 30°C to an OD_{600} of ~0.6. For sporulation, cells were grown either in Difco sporulation medium (DSM) overnight at 25°C or on DSM plates overnight at 30°C (Nicholson and Setlow, 1990). The signal was brighter when cells were grown on plates. The number of copies of the *lacO* cassette in strains AT18 and AT29 was increased by growing the strains on higher concentrations of chloramphenicol, which also resulted in a brighter signal.

Microscopy and Photography

Immunofluorescence was carried out as previously described (Harry et al., 1995; Pogliano et al., 1995), with the following modifications: the fixation used contained 0.06% glutaraldehyde (v/v) in 16% paraformaldehyde (v/v), and the cells were treated with lysozyme for 3 min. The cells were not treated with methanol or acetone. The polyclonal rabbit anti-GFP antibodies (Clonetech) were used at 1:4000 dilution, and the incubation time was 1 hr at room temperature. Anti-rabbit Cy3 secondary antibodies were used at a 1:200 dilution and incubated for 1 hr at room temperature, with wheat germ agglutinin-fluorescein (10 ng/ml) (Molecular probes) and 4,6-Diamidino-2-phenyloindole (DAPI) (2 μg/ml) (Sigma). Photomicrographs were taken as previously described (Harry et al., 1995; Pogliano et al., 1995), with the exception of the use of Fujichrome Provia400 film. Images were processed as previously described (Resnekov et al., 1996). For scoring, photomicrographs were taken using a Photometrics SenSys1400 Cooled CCD camera and a PowerMac9500 with IPLab Spectrum image processing software (version 2.5.6).

For observation of GFP signals, cells were resuspended in phosphate-buffered saline (pH 7.4) or water and placed on microscope slides. For better DNA staining, cells were lightly fixed as described for immunofluorescence above. The fix did not appear to affect the GFP signal, as similar results were observed without fixation. DAPI

was added to a final concentration of 2 μg/ml. Color photomicrographs were taken as previously described (Webb et al., 1995), with the exception of the use of Kodak Ektachrome 400X. Black and white photomicrographs were taken with the Photometrics SenSys1400 camera and a PowerMac9500 with IPLab Spectrum image processing software (version 2.5.6). Images were processed as previously described (Resnekov et al., 1996).

Acknowledgments

We thank S. Meyer for plasmid pSM79, D. Garsin for plasmid pDAG8-1, P. Stragier for plasmids pDG795 and pDG1832, and O. Resnekov for invaluable discussions and help with immunofluorescence experiments. C. D. W. is a predoctoral fellow of the National Science Foundation. This work was supported by NIH grants GM38035 to A. W. and GM18568 to R. L.

Received December 9, 1996; revised January 22, 1997.

References

Barton, N.R., and Goldstein, L.S. (1996). Going mobile: microtubule motors and chromosome segregation. Proc. Natl. Acad. Sci. *93*, 1735–1742.

Bone, E.J., Todd, J.A., Ellar, D.J., Sargent, M.G., and Wyke, A.W. (1985). Membrane particles from *Escherichia coli* and *Bacillus subtilis*, containing penicillin-binding proteins and enriched for chromosomal-origin DNA. J. Bacteriol. *164*, 192–200.

Cooper, S. (1991). The segregation of DNA and the cell surface. In Bacterial Growth and Division (San Diego, California: Academic Press), pp. 279–312.

Cutting, S.M., and Vander Horn, P.B. (1990). Genetic analysis. In Molecular Biological Methods for *Bacillus*, C.R. Harwood and S.M. Cutting, eds. (New York: John Wiley & Sons), pp. 27–74.

Driks, A., Roels, S., Beall, B., Jr., Moran, C.P., and Losick, R. (1994). Subcellular localization of proteins involved in the assembly of the spore coat of *Bacillus subtilis*. Genes Dev. *8*, 234–244.

Dubnau, E., Weir, J., Nair, G., III, Carter, H.L., Moran, C.P., and Smith, I. (1988). *Bacillus* sporulation gene *spoOH* codes for σ^{30} (σ^{H}). J. Bacteriol. *170*, 1054–1062.

Eberle, H., and Lark, K.G. (1966). Chromosome segregation in *Bacillus subtilis*. J. Mol. Biol. *22*, 183–186.

Fort, P., and Errington, J. (1985). Nucleotide sequence and complementation analysis of a polycistronic sporulation operon, *spoVA*, in *Bacillus subtilis*. J. Gen. Microbiol. *131*, 1091–1105.

Gartner, D., Geissendorfer, M., and Hillen, W. (1988). Expression of the *Bacillus subtilis xyl* operon is repressed at the level of transcription and is induced by xylose. J. Bacteriol. *170*, 3102–3109.

Harry, E., Pogliano, K., and Losick, R. (1995). Use of immunofluorescence to visualize cell-specific gene expression during sporulation in *Bacillus subtilis*. J. Bacteriol. *177*, 3386–3393.

Hiraga, S. (1992). Chromosome and plasmid partition in *Escherichia coli*. Annu. Rev. Biochem. *61*, 283–306.

Hirano, T., Mitchison, T.J., and Swedlow, J.R. (1995). The SMC family: from chromosome condensation to dosage compensation. Curr. Opin. Cell Biol. *7*, 329–336.

Hyman, A.A. (1995). Microtubule dynamics. Kinetochores get a grip. Curr. Biol. *5*, 483–484.

Ireton, K., Gunther, N.W., and Grossman, A.D. (1994). spo0J is required for normal chromosome segregation as well as the initiation of sporulation in *Bacillus subtilis*. J. Bacteriol. *176*, 5320–5329.

Jaacks, K.J., Healy, J., Losick, R., and Grossman, A.D. (1989). Identification and characterization of genes controlled by the sporulation regulatory gene *spoOH* in *Bacillus subtilis*. J. Bacteriol. *171*, 4121–4129.

Jacob, F., Brenner, S., and Cuzin, F. (1963). On the regulation of DNA replication in bacteria. Cold Spring Harbor Symp. Quant. Biol. *28*, 329–347.

LeDeaux, J.R., and Grossman, A.D. (1995). Isolation and characterization of *kinC*, a gene that encodes a sensor kinase homologous to the sporulation sensor kinases KinA and KinB of *Bacillus subtilis*. J. Bacteriol. *177*, 166–175.

Mobley, H.L.T., Koch, A.L., Doyle, R.J., and Streips, U.N. (1984). Insertion and fate of the cell wall in *Bacillus subtilis*. J. Bacteriol. *158*, 169–179.

Mohl, D.A., and Gober, J.W. (1997). Cell cycle-dependent polar localization of chromosome partitioning proteins in Caulobacter crescentus. Cell, this issue.

Moran, C.P., Lang, N., LeGrice, S.F., Lee, G., Stephens, M., Sonenshein, A.L., Pero, J., and Losick, R. (1982). Nucleotide sequences that signal the initiation of transcription and translation in Bacillus subtilis. Mol. Gen. Genet. *186*, 339–346.

Nicholson, W.L., and Setlow, P. (1990). Sporulation, germination and outgrowth. In Molecular Biological Methods for *Bacillus*, C.R. Harwood and S.M. Cutting, eds. (New York: John Wiley & Sons), pp. 391–450.

Pluta, A.F., Mackay, A.M., Ainsztein, A.M., Goldberg, I.G., and Earnshaw, W.C. (1995). The centromere: hub of chromosomal activities. Science *270*, 1591–1594.

Pogliano, K., Harry, E., and Losick, R. (1995). Visualization of the subcellular location of sporulation proteins in *Bacillus subtilis* using immunofluorescence microscopy. Mol. Microbiol. *18*, 459–470.

Resnekov, O., Alper, S., and Losick, R. (1996). Subcellular localization of proteins governing the proteolytic activation of a developmental transcription factor in *Bacillus subtilis*. Genes Cells *1*, 529–542.

Robinett, C.C., Straight, A.F., Li, G., Willhelm, C., Sudlow, G., Murray, A., and Belmont, A.S. (1996). In vivo localization of DNA sequences and visualization of large-scale chromatin organization using *lac* operator/repressor recognition. J. Cell Biol. *135*, 1685–1700.

Roels, S., and Losick, R. (1995). Adjacent and divergently oriented operons under the control of the sporulation regulatory protein GerE in *Bacillus subtilis*. J. Bacterol. *177*, 6263–6275.

Sandler, N., and Keynan, A. (1988). Membrane binding and release of *Bacillus subtilis* DNA as a function of the cell cycle. J. Gen. Microbiol. *134*, 1155–1163.

Setlow, B., Magill, N., Febbroriello, P., Nakhimovsky, L., Koppel, D.E., and Setlow, P. (1991). Condensation of the forespore nucleoid early in sporulation of *Bacillus* species. J. Bacteriol. *173*, 6270–6278.

Sharpe, M.E., and Errington, J. (1995). Postseptational chromosome partitioning in bacteria. Proc. Natl. Acad. Sci. USA *92*, 8630–8634.

Sharpe, M.E., and Errington, J. (1996). The Bacillus subtilis *soj-spo0J* locus is required for a centromere-like function involved in prespore chromosome partitioning. Mol. Microbiol. *21*, 501–509.

Straight, A.F., Belmont, A.S., Robinett, C.C., and Murray, A.W. (1996). GFP tagging of budding yeast chromosomes reveals that protein–protein interactions can mediate sister chromatid cohesion. Curr. Biol. *6*, 1599–1608.

Sun, D., Fajardo-Cavazos, P., Sussman, M.D., Tovar-Roja, F., Cabrera-Martinez, R.-M., and Setlow, P. (1991). Effect of chromosome location of *Bacillus subtilis* forespore genes on their *spo* gene dependence and transcription by $E\sigma^{F}$: identification of features of good $E\sigma^{F}$-dependent promoters. J. Bacteriol. *173*, 7867–7874.

Wake, R.G., and Errington, J. (1995). Chromosome partitioning in bacteria. Annu. Rev. Genetics *29*, 41–67.

Webb, C.D., Decatur, A., Teleman, A., and Losick, R. (1995). Use of green fluorescent protein for visualization of cell-specific gene expression and subcellular protein localization during sporulation in *Bacillus subtilis*. J. Bacteriol. *177*, 5906–5911.

Woldringh, C.L., Huls, P., Pas, E., Brakenhoff, G.J., and Nanninga, N. (1987). Topography of peptidoglycan synthesis during elongation and polar cap formation in a cell division mutant of *Escherichia coli* MC4100. J. Gen. Microbiol. *133*, 575–586.

Wu, L.J., and Errington, J. (1994). *Bacillus subtilis* SpoIIIE protein required for DNA segregation during asymmetric cell division. Science *264*, 572–575.

Wu, L.J., Lewis, P.J., Allmansberger, R., Hauser, P.M., and Errington, J. (1995). A conjugation-like mechanism for prespore chromosome partitioning during sporulation in *Bacillus subtlis*. Genes Dev. *9*, 1316–1326.

Youngman, P., Perkins, J.B., Losick, R. (1984). Construction of a cloning site near one end of TN*917* into which foreign DNA may be inserted without affecting transposition in *Bacillus subtilis* or expression of the transposon-borne *erm* gene. Plasmid *12*, 1–9.

Zuber, P., and Losick, R. (1983). Use of a *lacZ* fusion to study the role of the *spoO* genes of *Bacillus subtilis* in developmental regulation. Cell *35*, 275–283.