Replication and segregation of an *Escherichia coli* chromosome with two replication origins

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Characterized bacteria, unlike eukaryotes and some archaea, initiate replication bidirectionally from a single replication origin contained within a circular or linear chromosome. We constructed *Escherichia coli* cells with two WT origins separated by 1 Mb in their 4.64-Mb chromosome. Productive bidirectional replication initiated synchronously at both spatially separate origins. Newly replicated DNA from both origins was segregated sequentially as replication progressed, with two temporally and spatially separate replication termination events. Replication initiation occurred at a cell volume identical to that of cells with a single WT origin, showing that initiation control is independent of cellular and chromosomal oriC concentration. Cells containing just the ectopic origin initiated bidirectional replication at the expected cell mass and at the normal cellular location of that region. In all strains, spatial separation of sister loci adjacent to active origins occurred shortly after their replication, independently of whether replication initiated at the normal origin, the ectopic origin, or both origins.

Like most bacteria, *Escherichia coli* harbors a single circular chromosome within which replication is initiated at a single origin, oriC, and progresses bidirectionally toward the diametrically opposite replication terminus region (ter). The *E. coli* replication machinery assembles at the oriC, close to midcell at replication initiation, and in minimal medium, the two replisomes track independently around the chromosome (1). The spatial separation of many newly replicated sister genetic loci to opposite cell halves occurs sequentially 5 to 20 min after replication (1–6). Similarly, sequential replication-segregation has also been described in *Caulobacter crescentus* (7, 8) and *Vibrio cholerae* (9). Interlinking of newly replicated sisters (i.e., precatenation) may be responsible for the 5- to 20-min delay between replication and separation, because overexpression of topoisomerase IV, which plays a key role in decatenation, led to an approximate threefold decrease in the sister colocalization period of a locus 15 kb to the left of oriC (10). Consistent with this, inhibition of topoisomerase IV led to wholesale defects in sister chromosome segregation (10). Nevertheless, genetic loci in at least two discrete 150 kb regions, approximately 130 kb and 380 kb to the right of oriC, exhibited a further approximately 18 min delayed separation compared with neighboring loci (2–4). The mechanism that leads to this delayed separation of some sister loci on the right chromosome arm remains unclear and may not be linked to decatenation. Nevertheless, it was associated with an abrupt global transition in nucleoid morphology that may play a key role in sister nucleoid separation (2, 3).

In each of the three domains of life, replication is tightly regulated, so that no origin normally fires more than once per cell cycle. Because cell generation time can be modulated, overall rates of DNA synthesis within an organism or cell type must also be regulated. In *Drosophila* embryogenesis, as in other eukaryotes, a decreased S phase is usually accommodated by firing from an increased number of active origins, with the DNA synthesis rate of any pair of sister replication forks remaining constant (11). Similarly, in *E. coli*, overall DNA synthesis can be increased to reduce the generation time by increasing the number of active replication forks in the cell. For example, sister replisomes derived from a single initiation event take at least 40 min to replicate the whole chromosome (i.e., C-period), and cell division then follows approximately 20 min (i.e., D-period) after the completion of replication. When the generation time is shorter than the sum of C- and D-periods, initiation occurs synchronously at sister origins within a chromosome that is already undergoing replication (12–15).

In *E. coli*, the ATP-bound form of the abundant DnaA initiator protein controls replication initiation at oriC. A range of regulatory mechanisms ensures precise and controlled initiation timing by modulating DnaA binding and action on DNA (reviewed in refs. 16–18). Early data led to a model in which initiation occurs at a constant mass per chromosome origin (19), although the demonstration that cells containing oriC plasmids initiated DNA synthesis synchronously at the same mass as their plasmid-free parent, along with other data, showed that this model could not be strictly true (20, 21; reviewed in refs. 22, 23). Nevertheless, the mechanistic relationship, if any, between initiation, cell growth and mass remains unclear.

The processes that contribute to *E. coli* chromosome organization and segregation are poorly understood. A range of proteins and mechanisms that could facilitate chromosome segregation has been proposed, although no consensus mechanistic view has emerged. It has been proposed also that *E. coli* chromosomes could segregate spontaneously by using a self-avoidance mechanism driven by entropy (24, 25). In contrast, in *Bacillus subtilis* and *C. crescentus*, chromosome tethering mechanisms attach specific chromosome regions to cell poles (26–28), whereas dedicated partition systems facilitate chromosome segregation (reviewed in ref. 29). In *E. coli*, low copy number plasmids use similar partition systems (29), but none that contribute to *E. coli* chromosome segregation have been characterized. Independent tracking of sister replisomes around the chromosome could contribute directly to segregation (1).

To gain insights into the processes that govern replication and chromosome segregation, we analyzed *E. coli* cells with two identical functional replication origins separated by approximately 1 Mb (oriC-oriZ) and cells containing just the ectopic origin (oriZ), and compared them with WT cells (oriC). In oriC-oriZ cells, replication initiation occurred synchronously at both origins, which are located at the normal separate cellular locations of the loci associated with the origins. Genetic loci adjacent to oriC and to the insertion site of the ectopic oriC segregated sequentially as...
replication progressed, with two temporally and spatially separate replication termination events. Synchronous replication initiation at both origins occurred at a cell mass identical to that of WT cells with a single oriC, showing that the concentration of oriC within a chromosome does not influence the timing of replication initiation. The initial segregation pattern of the pairs of sister origins, in which sisters of a given locus lay side-by-side, is reminiscent of sister origin segregation in fast growing cells, when sisters only segregate to opposite cell halves and then to daughter cells, one to two generations after the initial segregation. Such behavior, which is incompatible with faithful chromosome segregation in the two-origin strain, was switched to a permissive pattern at the time of completion of replication of the smaller chromosome segment between oriC and oriZ. Cells with two origins or just the single ectopic oriC grew with relatively normal growth rates and cell cycle parameters, thereby demonstrating the robustness and adaptability of E. coli chromosome processing.

**Results**

**Growth, Size, and Cell Cycle Parameters of E. coli with Two Distant Replication Origins and a Single Ectopic Origin.** A copy of the 5.1-kb region containing oriC was inserted approximately 1 Mb away into the intergenic region at 344 kb on the E. coli genetic map, 21 kb upstream of lacZ (Fig. 1A). We name the ectopic origin locus oriZ. The oriC-oriZ strain, containing two copies of oriC, was further manipulated to have the 5.1-kb oriC region deleted from its original locus so that the only replication origin is at oriZ (Fig. 1B). We then characterized the cell cycle, replication, and segregation features of the oriC-oriZ strain with those of the oriZ strain and the oriC AB1157 parent. The cellular localization of oriC and oriZ was followed by using fluorescently labeled repressors bound to lacO and tetO operator arrays at ori1, 15 kb from oriC on the left replication arm, and at R2, 21 kb downstream of oriZ (6). Transposition of a smaller 1.2-kb oriC region (Fig. 1A) to the same ectopic position gave strains with indistinguishable properties to those described herein.

The doubling times and viabilities of all three strains were similar in minimal medium and in rich medium (Fig. 1B). Thus, the presence of two origins, or a single ectopic origin, in a single chromosome, had no substantial effect on generation time. We observed no loss of viability or abnormal cell morphology for any of the three strains, indicating that E. coli tolerates well the introduction of an additional ectopic origin and the subsequent duplication of the WT origin. We constructed oriC-oriZ and oriZ strains many times independently and found no evidence that any of the phenotypes we observed resulted from the accumulation of suppressor mutations.

We further characterized the three strains by examining cell size and cell cycle features. Flow cytometry profiles allowed estimation of the sum of C- plus D-period for each of the strains; and the time of initiation of replication and the C-period, as assessed by flow cytometry were in broad agreement and additionally showed that the cell volume arithmetically as $t$ equal to the sum of B-, C-, and D-periods under our growth conditions. Cell volumes (in $\mu$m$^3$) were measured by flow cytometry and direct microscopic measurement. During time-lapse analysis, cell doubling time on the agarose pads increased by 13% (oriC and oriC-oriZ strains) and 14% (oriZ); with a proportional increase in B-, C-, and D-periods, compared with growth in liquid medium. The values given have been compensated for this and reflect liquid growth values. The viabilities, determined as colony forming units per A600 were 1.95 x $10^{10}$ (oriC) and 1.85 x $10^{10}$ (oriC-oriZ and oriZ).

**Fig. 1.** (A) Genetic map of the WT E. coli chromosome, with the left replication in blue and the right replication in orange. A zoom of the origin region shows the minimal initiation site oriC (258 bp) and its genetic context alongside the 5.1-kb and 1.2-kb DNA region, the segments that were relocated and/or deleted. oriZ (at 344 kb on the E. coli genetic map) is the site where the ectopic origin was inserted. The red and blue stars respectively indicate the insertion position of ori1 (lacO, at 3,908 kb) and R2 (tetO, at 366 kb) operator arrays used for chromosome localization. Replication termination sites (black dumbbell shapes), RNA operons (black arrows), DnaA-binding DARS1 and DARS2 regions (812 kb and 2,967 kb, respectively), and the DnaA-binding cluster data (3,494 kb) are shown, as is the dif site at which XerCD-FtsK recombination occurs. (B) Chromosome structure and representative newborn cells, with fluorescently marked ori1 (red) and R2 (blue) loci, of all three strains analyzed in detail. The replication arms inferred from the position of origins and terA and terC sites are represented by light gray arrows. Dashed lines indicate where replication and transcription are head-on. Lower: Doubling times (min; $\tau$) in rich and minimal media, cell cycle parameters from flow cytometry (Fig. S1), and microscopy characterization (Figs. 3 and 4 and Fig. S2). B-period (min) is the time from birth to initiation of DNA replication, as measured by appearance of the replication marker Ypet-DnaN. C-period (min) is the time of DNA synthesis assessed by replication appearance to disappearance and by flow cytometry (C- plus D-period). D-period (min), the time from termination of DNA synthesis to division, was assessed by flow cytometry (C- plus D-period) and growth, Size, and Cell Cycle Parameters of E. coli with Two Distant Replication Origins and a Single Ectopic Origin.
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Synchronous Initiation of Replication from Both Origins. To test
with two origins is identical to that of cells with one origin.
oriC
and perturbed initiation timing (31, 32). Importantly, we have
appearance, as observed by time-lapse microscopy. ori1 (lacO) was visualized by
using Lac-mCherry (red); R2 (tetO) was visualized by using TetR-CFP (blue).

tributions for all three strains were very similar, with replication
initiation occurring at similar cell volumes, and at the same
time within the cell cycle. The flow cytometry analysis also showed that
during growth in minimal glycerol medium, all three strains ini-
tiated and completed replication in the same generation.

Taken together, these observations show that the regulation of
E. coli replication and of the cell cycle is maintained regardless of
the presence of two identical functional origins or replication
from an ectopic origin. This contrasts with the situation in B. subtilis, in which relocation of oriC to sites more than 250 kb
distant from the normal position led to asynchronous initiation
and perturbed initiation timing (31, 32). Importantly, we have
demonstrated the cellular and chromosomal concentration of
oriC is not a determinant in control of replication initiation, as
originally proposed (19), as cell mass at initiation of the strain
with two origins is identical to that of cells with one origin.

Synchronous Initiation of Replication from Both Origins. To test
whether the oriC-oriZ strain initiates replication from both origins
and whether each of these function in every cell generation, we
used fluorescence microscopy to analyze replisome assembly
and localization at each of the origins. First, we tested whether oriZ
fires synchronously with oriC at initiation in a dnaC2 temperature-
sensitive strain, which allows synchronous replication initiation
in a large population of cells (33). Exponentially growing cells at
30 °C were shifted to 37 °C at an optical density A₆₀₀ of approximately 0.1 for 2 h to allow completion of ongoing rounds of
replication, after which they were shifted back to 30 °C to allow
replication initiation. Intracellular positions of ori1, R2, and
replisomes were then analyzed 10 min after temperature down-
shift, when replication reinitiates (Fig. 2A). A single replisome
focus colocalized with ori1 in oriC cells and with R2 in oriZ cells
(82% and 86% of cells, respectively); the remaining cells were
largely without a replisome focus (15% and 10% for the two
strains, respectively), indicating that the replication has not yet
initiated in those cells. The fewer than 3% of cells that had two
replisome foci in these two strains had presumably undergone
spatial separation of sister replisomes within the 10-min time
frame (1).

In contrast, in the oriC-oriZ two-origin strain, two replisome
foci appeared and colocalized with each origin in 67% of cells
within 10 min of initiation (Fig. 2B). Furthermore, the replisomes
appeared at the normal WT intracellular positions of ori1 and R2,
confirming that independently acting replisomes assemble on
origins irrespective of their cellular location, rather than being
part of a replication factory that recruits origins to it (1, 30).

We were concerned that an accumulation of DnaA-ATP ini-
itiator protein in dnaC2 cells during incubation at the restrictive
temperature could have led to initiation at both origins in the
oriC-oriZ strain, whereas steady-state cells might not accumulate
sufficient DnaA-ATP to allow productive initiation at both or-
gins. Therefore, we analyzed replisome assembly at the two or-
gins in exponentially growing cells by using time-lapse (Fig. 2B)
and snapshot analysis (Fig. S2B). Time-lapse experiments dem-
onstrated that replisome foci appeared synchronously at each of
the two spatially separate origins within a 5-min time interval in
68% of initiation events in oriC-oriZ cells (Fig. 2B, green arrows);
replisome appearance inevitably led to active replication and
subsequent segregation of the newly replicated loci adjacent to
each of the origins. In contrast, synchronous appearance of two
replisome foci in oriC or oriZ cells was never observed.

Snapshot analysis of replisome foci confirmed these conclusions
(Fig. S2B). Younger cells, between 2 and 2.5 μm in length, in which
initiation happens, had an overrepresentation of cells with two or
more replisome foci in the oriC-oriZ strain (51%), compared with
WT cells (28%) or cells containing just oriZ (13%). A concomitant
reduction in the proportion of single replisome focus cells in the
oriC-oriZ strain was also evident. These data confirm that firing of
both oriC and oriZ occurs within individual steady state
oriC-oriZ cells.

To show that initiation at both origins in oriC-oriZ cells led to
productive replication, newly replicated DNA was labeled with 5-
ethyl-2′-deoxyuridine (EdU) after synchronous initiation in
dnaC2 cells (SI Materials and Methods and Fig. S2C). Visualization
of the label showed that the majority of oriC-oriZ cells had
two spatially separate fluorescent foci (54% after 8 min and 64%
after 15 min of replication), whereas most oriC cells had a single
focus (87% and 81%, respectively, at the two time points),
confirming that replication in two-origin cells occurs at both spatially
separate origins.

Replication Fork Progression in oriC-oriZ and oriZ Cells. Because the
presence of a second replication origin in oriC-oriZ cells, or
changing the origin position in oriZ cells was likely to modify
chromosome replication and subsequent segregation, we then
studied the patterns of replication fork progression in these strains.
Synchronous initiation at oriC and oriZ in oriC-oriZ cells would
therefore be expected to lead to cells with four replisomes at four
active forks until replication fork meeting and termination within
the approximately 1 Mb segment, approximately 13 min after
initiation, if replication of both small replichores occurs un-
implied (Fig. 1A). Consistent with this, time-lapse analysis showed that cells with at least three replisomes were only present for as long as 20 min after initiation (Fig. 3A and B). Cells with four replisomes were rare (<2%) in both the time-lapse and snapshot analyses (described later), an observation that is not surprising, as it takes at least 5 min for independent replisomes to become spatially separate (1). We conclude that cells with at least three replisomes are undergoing replication of both chromosome segments that lie between oriC and oriZ and that replication of the smaller, approximately 1 Mb segment occurs without significant delay. This indicates that replication at both forks in the smaller segment contributes to duplication of the segment, and therefore that the counterclockwise (CCW) fork is not substantially impeded by “head-on” rRNA transcription (Fig. 1A).

Indeed, if the CCW fork from oriZ was regularly stalling at the head-on rRNA cistron, we might have observed a significant number of four-replisome cells. The observation that the C-period of oriC-oriZ cells was almost identical to that of oriC cells is not surprising, given that a single fork replicates approximately 2.3 Mb in both strains. The time-lapse conclusions are supported by snapshot analysis, which revealed that 14% of 2,472 cells examined had at least three replisome foci (Fig. 4A).

In oriZ cells, we observed an almost twofold reduction in the proportion of two-replisome cells compared with oriC cells (snapshots, Fig. S2B). Furthermore, two-replisome cells were absent for the last 31 min of the C-period of the majority of oriZ cells, compared with the last approximately 10 min for oriC cells (time-lapse, Fig. 3). Analysis of replisome number and position with respect to R2 in oriZ cells showed no evidence for replication initiating at sites other than oriZ in oriZ cells. These observations indicate strongly that replication termination does not occur diagonally opposite to oriZ, with equal-sized CCW and clockwise (CW) replisomes. Rather, it is consistent with the view that the CW fork reaches Tus-bound terC or a subsequent Tus-bound ter site (Fig. 1B), and eventually disassembles there. The CCW fork then would complete replication when it encounters the stalled fork at ter. We would therefore have anticipated that the C-period would be increased at least 50% from the approximately 56 min of oriC cells to more than 84 min, in the case of termination at terC, as a single fork would now need to replicate approximately 3.3 Mb of DNA. The more modest 17-min increase (30%) in C-period we observed could be explained by a compensating increased fork replication rate as a consequence of the CCW fork having available twice the amount of the normally limiting dNTPs for synthesis during much of the C-period (e.g., refs. 34, 35). Alternatively, CW forks could pass through one or two of the ter sites following terC, leading to marginally more equal replisomes, as proposed for strains carrying inversions that unbalance replisome size (36). Other inversions encompassing oriC, which generated a replisome unbalance comparable to that proposed for oriZ cells, displayed viability defects (37), unlike the situation in oriZ cells. Nevertheless, these inversions additionally disrupted macromolecular organization, the integrity of which is important for normal cell viability (37, 38).

**Patterns of Genetic Locus Segregation Are Determined by Replication.**

We then examined how the positioning and segregation of ori1 and R2, adjacent to oriC and the oriZ insertion site, respectively, behaved with respect to replication fork progression in the three strains. Snapshot analysis was used to assess the behavior of large populations, and time-lapse analysis followed the behavior of the replisome and chromosome loci through time. The time-lapse analysis was facilitated by the use of an automated custom particle-tracking algorithm (Materials and Methods).

We observed that the time between the separation of sister ori1 loci and sister R2 loci was strictly dependent on the replication program of the chromosome. In oriC cells, replication of ori1 is expected to occur approximately 0.4 min after replication initiation, and we observed sister ori1 locus separation approximately 7.5 min after initiation, consistent with our earlier estimate of the sister origin colocalization period (1, 10). The R2 locus, 1,073 kb away from oriC, was expected to replicate approximately 26 min after replication initiation. Consistent with this, the time-lapse analysis showed that sister R2 loci separated, on average, ap-
approximately 27.5 min after initiation (Fig. 3B and Fig. S2A). In oriC-oriZ cells, synchronous replication initiation at both origins was followed by spatial separation of newly replicated sister ori1 and R2 loci within the same time-lapse frame (0–5-min difference), or with a difference of one time-lapse frame (5–10 min), showing that replisome appearance at each origin is followed by functional replication and subsequent sister separation a few minutes later (Figs. 3B and 4A and Fig. S2).

Similarly, we observed in oriZ cells that newly replicated sister R2 loci separate approximately 17.5 min earlier than ori1 sisters (Fig. 3B and Fig. S2A and E); replication initiating at oriZ is expected to reach ori1 approximately 26 min later. Therefore, it appears that the progress of the CCW fork in oriZ cells is not substantially impeded by head-on transcription from the five ribosomal cistrons that this fork will inevitably encounter (Fig. 1A). We also note that in both oriC and oriZ cells, the sister loci proximal to the origin show a delay in their spatial separation compared with the distal locus.

Snapshot analysis supported the aforementioned observations: the average number of ori1 and R2 foci per cell were, respectively, 1.5 and 1.3 (oriC); 1.3 and 1.3 (oriC-oriZ); and 1.5 and 1.7 (oriZ; Fig. S2E). Therefore, the relative times of spatial separation of sister ori1 and R2 loci followed the order of their replication, with the first region to be replicated being the first to be segregated, as was shown previously for the same loci in WT cells (4, 6). Nevertheless, by displacing and/or duplicating origins, we are additionally able to conclude that the time of segregation of these sister chromosome loci is determined by the cell’s replication program, rather than by intrinsic properties of chromosomal regions, or by a replication-independent segregation machinery.

Next, we examined ori1 and R2 locus positioning within cells throughout the cell cycle. Newborn cells of all three strains, containing nonreplicating chromosomes, had identical locus positioning, with ori1 close to midcell and R2 toward the quarter position (Fig. 1B and Fig. S2F). Therefore, changing origin position and number does not influence the cellular position of the genetic loci tested in nonreplicating chromosomes. In all three strains undergoing steady-state growth, ori1 was replicated close to midcell and ori1 sisters segregated toward the quarter positions, where they stayed until cell division (Fig. S2A and E). Therefore, cellular ori1 positioning is not a consequence of ori1 being adjacent to an active origin.

Cellular positioning of single R2 loci in oriC-oriZ and oriZ cells was conserved when compared oriC cells, irrespective of whether the loci represented unreplicated or replicated loci. Nevertheless, the relative position of given R2 sisters in individual oriC-oriZ and oriZ cells, and their relationship to ori1 sisters, showed some differences, compared with oriC cells. In approximately 80% of sister oriC cells, one R2 sister positions closer to an old pole and one closer to a new pole, thereby generating the observed translational symmetry (<R2 ori1 ori1 R2> vs. <R2 ori1 ori2 R2>; Fig. 1F, 39). In both oriC-oriZ and oriZ cells, there was an increased tendency for the sister R2 loci to locate proximal to the old pole, thereby generating an increased proportion of <R2 ori1 ori1 R2> cells in snapshots (8% for oriC, 29% for oriC-oriZ, and 34% for oriZ; Fig. S2D). The increased migration apart of sister R2 loci when they are adjacent to an active origin, which was slightly more pronounced in oriZ cells compared with oriC-oriZ cells, was directly observed in time-lapse analysis (Fig. S2A and D). The reason for this increased tendency of R2 sisters to move to the old poles, when an adjacent oriZ origin is active is unclear, although it is reminiscent of sister oriC bipolar migration to the outer poles when a functional Structural Maintenance of Chromosomes complex, MukBEF, is absent (40).

**Switch in Locus Segregation in oriC-oriZ Cells.** When we examined ori1 and R2 positioning in oriC-oriZ cells harboring three or four replisomes, most of which are expected to have four replisomes active, we found evidence for a dramatic switch in segregation pattern as replication of the smaller, approximately 1 Mb segment is completed. In more than 90% of cells with three or more replisomes in which sisters of at least one genetic locus had sep-
ated (Fig. 4A, lines b–g), sister ori1 and/or R2 loci have not separated into opposite cell halves (Fig. 4A, lines b–d), and are therefore not positioned to allow their segregation into prospective daughter cells. In contrast, by the time replication is complete as judged by the disappearance of replisome foci (13% of 1,894 cells examined), all cells have adopted a configuration in which sisters now occupy separate cell halves and are thereby permissive for productive segregation (Fig. 4E, lines e–g). In cells with a single functional origin on one chromosome, the configuration in line d is hardly ever observed. Indeed, the initial configuration of sister loci, in which they lie adjacent to each other, is reminiscent of the situation in fast-growing cells with overlapping replication cycles, in which synchronous initiation at two or four origins, leads to sister pairs only being segregated to daughter cells one to two generations after their replication (Fig. 4B). The switch from an initial <ori1 ori1 R2 R2> pattern to permissive <ori1 R2 ori1 R2> or <R2 ori1 R2 R2> patterns appears to wait for completion of replication of the chromosome segment lying between oriC and oriZ, as judged by the number of replisome foci. This switch in configuration was validated by time-lapse analysis (Fig. S2). We propose that the switch to a permissive pattern becomes possible only when a topological restraint to complete segregation, imposed by the incomplete replication of the small segment, is removed by the completion of replication. In fast-growing cells, in which replication initiates synchronously at two or more origins, such a constraint may play a crucial role in ensuring that newly replicated sisters are prevented from immediately segregating to opposite cells halves (Fig. 4B). Our own work has not revealed a similar “unlocking” of a topological restraint in replicating oriC cells, as judged by the delayed spatial separation of specific sister loci. Nor is it necessary, a priori, to invoke such an unlocking mechanism. Nevertheless, a delayed (~18 min compared with most loci) spatial separation of sister loci in two 150 kb regions in the right chromosome arm, approximately 130 kb and 380 kb from oriC, has been observed by Kleckner, Austin, and their colleagues (24–27). The eventual separation of these sister loci was associated with an abrupt “splitting” of the nucleoid into a bilobed structure, a process proposed to play role in the segregation process (3).

Discussion

This work provides insight into control of replication initiation and the processes that influence the positioning and segregation of E. coli chromosome loci and the replication machinery. By characterizing replication initiation in cells containing two distinctly separated yet functional replication origins, we have demonstrated that neither cellular nor chromosomal concentration of oriC is a determinant in control of replication initiation, as originally proposed (19), as cell volume at initiation of the oriC-oriZ strain with two origins is identical to that of oriC WT and oriZ cells. Although subsequent work, most of which used oriC-carrying plasmids, showed that initiation mass/oriC copy is not strictly constant (20–23), this is the first study of which we are aware in which cells with more than one WT origin within a bacterial chromosome has been extensively characterized.

We propose that, at all growth rates, total chromosomal DNA concentration in a cell at initiation, rather than oriC concentration, is a key factor in control of initiation by DnaA-ATP, because chromosomal DNA titrates most of the more than 1,000 DnaA-ADP and DnaA-ATP molecules, with only a small fraction being oriC-associated (reviewed in ref. 41). This hypothesis is not only consistent with the observation that the insertion of an additional origin sequence into the E. coli chromosome has no significant effect on the volume at which initiation occurs, but is broadly consistent with the calculated chromosomal DNA concentrations at which synchronous initiation at one, two, or four sister origins occurs [1:1:0.8, respectively (14); see http://simon.bio.uva.nl/cellcycle/index.html]. Furthermore, our own empirical data for slow- and fast-growing cells are also broadly consistent with initiation at a constant chromosomal DNA concentration (Fig. S1C). We note that the relative positioning of functional origins with data1 and DARS chromosomal sequences, which bind DnaA and act in normal initiation control (Fig. 1A) (16, 41), is similar in each of the three strains. If regulatory inactivation of DnaA (16, 18) stimulates hydrolysis of DNA-bound DnaA-ATP at the fork as replication proceeds, the ratio of DnaA-ATP to DnaA-ADP should increase during D-periods. Therefore, irrespective of growth rate, the increase in concentration of DnaA-ATP associated with reduced DNA synthesis during a D-period may be a key event in controlling reinitiation, as well as being the time during which the final stages of chromosome segregation and cell division occur.

The demonstration that replication in oriC-oriZ cells is initiated synchronously at spatially separate origins, residing at the expected cellular positions of ori1 and R2, confirms that E. coli replication does not occur in replication factories containing sister replisomes in cells that initiate and complete replication in the same generation (1). Synchronous initiation at both origins is followed by productive replication and separation of sister ori1 and R2 loci, as judged by fluorescent labeling of newly replicated DNA at initiation and by sister foci separation. Indeed, separation of newly replicated sister ori1 and R2 loci occurred soon after locus replication irrespective of whether replication initiated at oriC, oriZ, or both origins synchronously. As most other chromosomal loci appear to behave like ori1 and R2 (24–27), the data also extend support for the hypothesis that the timing of chromosome locus segregation is determined largely by the timing of replication, rather than by some intrinsic property of chromosome content or organization, perhaps associated with a dedicated segregation machinery. Nevertheless, delayed separation of a few specific regions may be instrumental in catalyzing nucleoid splitting (2).

The cellular location of the normal oriC region is independent of the presence of a functional origin, whereas inserting a functional origin into an ectopic locus does not perturb the cellular position of that locus in nonreplicating cells. Similarly, in B. subtilis, the positioning of the origin region remains the same after deletion of the initiation sequences (32), whereas insertion of oriC into a plasmid does not alter the plasmid’s cellular location in E. coli (42). In chromosomal organization in oriC, oriC-oriZ, and oriZ cells an intrinsic property of the chromosome and its replication pattern, or are there external factors involved in locus position and origin behavior, as in C. crescentus or during sporulation in B. subtilis? We have demonstrated functional expression of C. crescentus PopZ and ParB in E. coli and have shown that PopZ localizes to poles, where it can capture ParB in the absence of chromosomal parS sites (Fig. S3) (27, 28). Nevertheless, oriC-proximal parS sites bound by ParB, were not recruited to the poles by PopZ (Fig. S3). Therefore, it is not trivial to alter E. coli chromosome organization or behavior by introducing a polar tethering system. These observations indicate again that chromosome organization is not readily influenced by extrinsic factors. In oriC cells, the location of oriC close to midcell could arise simply from the fact that replication “layers” equal amounts of DNA on either side of the newly replicated origins, because the replication arms (i.e., replisomes) are of almost identical size. Similarly, in oriZ cells, if replication terminates within the normal ter, layering of two very different sized replisomes, of approximately 3 Mb and 1 Mb (Fig. 1B), could have led to the R2 locus remaining at approximately the cell quarter position. Alternatively, a process other than replication may contribute to genetic locus positioning.

We observed no overriding problems in replicating through strong convergent transcription from the ribosomal RNA cistrons. In rich and minimal medium, generation times were similar to that of the WT strain, with no obvious filamentation or morphological
abnormalities. The cell cycle parameters, measured in minimal medium, were also comparable despite the changed replication programs. In oriZ cells, it appears that the single CCW replicates through all five rRNA cistrons head-on, without compromising substantially the time of segregation of sister oriZ loci, or the overall C-period. Similarly, strains carrying inversions that include multiple rr operons were shown to be fully viable in rich and minimal media, as long as a WT complement of helicases that facilitate replication fork progression through potential “roadblocks” was present (37, 43). We note that, in B. subtilis, head-on collision between rRNA transcription and replication have little effect on generation time in minimal medium (despite a ~30% decrease in overall DNA elongation rate), but resulted in a greater than threefold increase in generation time in Luria Broth (LB) and as much as a sevenfold increase if combined with unbalancing of replicores (44). The apparent differences between E. coli and B. subtilis in dealing with head-on collisions between DNA polymerase and RNA polymerase may be because their DNA replication machineries have different compositions (45–47), and they use different nonreplicative helicases for removing roadblocks on DNA (43). Additionally, we showed that replication can effectively terminate in regions of the chromosome distant from ter sites and dfr, showing that the converging replication fork machineries, along with other chromosome processing activities, can handle the final stages of replication and decatenation at sites distant from the normal ter and from the region of the cell where division will next occur. This is consistent with the demonstration that decatenation by topoisomerase IV occurs as replication proceeds around the chromosome (10).

We have demonstrated a dramatic robustness and flexibility of E. coli chromosome replication and segregation. Under laboratory conditions, cells can accommodate at least two functional origins, or an ectopic origin, without obvious detriment. This is despite oriC-oriZ and oriZ cells having altered replication-segregation patterns, with replication termination occurring distant from the normal termination sites and head-on collisions with the high levels of transcription from rRNA cistrons. Other work has also shown that E. coli can also accommodate a lifestyle with linear chromosomes (48), or with chromosomes carrying large inversions (36–38). Nevertheless, over evolutionary time, natural selection has favored gene and gene expression organizations that show common features when related to the normal single replicons of other bacteria (e.g., replication origins (44, 49)). Key genes tend to be located close to replication origins so their cellular concentration remains constant irrespective of growth rate. Furthermore, strong and/or long transcriptional units are arranged to avoid head-on collisions with replication. In eukaryotes, too, replication is prevented from head-on collisions with RNA transcription by the presence of specific termination barriers (reviewed in ref. 50).

**Materials and Methods**

**Bacterial Strains and Plasmids and Their Propagation.** Derivatives of E. coli K12 AB1157 growing in M9 glycerol (0.2%) supplemented with essential nutrients were used unless otherwise stated (6, 51). Ampicillin (100 μg/mL), kanamycin (25 μg/mL), gentamycin (15 μg/mL), hygromycin (50 μg/mL), and chloramphenicol (20 μg/mL) were added when required. Visualization of genetic loci using lacO and tetO arrays was as described by Wang et al. (6, 51). Fluorescent fusions to LacI and TetR were expressed at low levels to minimize perturbation of DNA replication and segregation (6, 51).

**Flow Cytometry.** Flow cytometry was performed as described (36). Cells were treated with rifampicin (300 μg/mL) and cephalexin (100 μg/mL) for 4 h for runout experiments before fixation using ethanol (75%) and staining using Syto16 (0.2 μM). Analysis of the DNA content per cell was performed with a FACSscan flow cytometer (Becton). Waseal software was used for plotting and analysis.

**Microscopy.** Snapshot images were taken from exponentially growing cells (A600 0.2). For time-lapse acquisition, cells were transferred from liquid culture to a slide mounted with 1% agarose in the same growth medium, and incubated at required temperature using an incubation chamber. Images were captured with a 5-min time interval. Microscopy was carried out on a Nikon Eclipse TE2000-U microscope equipped with a Photometrics Cool-Snap HQ CCD or a QuantEM camera. All images were taken and analyzed using MetaMorph 6.2 and ImageJ software.

**Automatic tracking of the fluorescent signal was performed using a custom MATLAB routine adapted from the from the feature point tracker of Sbalzarini and Koumoutsakos (53). Identification of the cell poles was performed interactively by the users because automatic identification in bright-field images could not be reliably achieved. Three image sequences were then tracked: one consisting of artificial cell center “particles” and the other two the GFP and YFP channels containing the foci. As many as two foci per channel were assigned to each cell on the basis of their proximity to the cell center line running between the two poles. Output from the program consists of a table of coordinates for the cell poles, cell centers, and loci position, and, for each cell selected by the user, a kymograph summarizing the chromosome loci dynamics.

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Fluorescent Labeling of Newly Replicated DNA in Synchronized Cells.

dnaC2 strains carrying only WT oriC and oriC-oriZ, respectively, were grown in M9 glycerol medium at 30 °C to an OD of approximately 0.1 and synchronized at 42 °C for 3 h. EdU and dAdenosine was added to a final concentration of 20 and 100 μg/mL respectively and then cultures were transferred to 30 °C. Cells were fixed by adding cold methanol to a final concentration of 72% at 8 or 15 min after temperature shift. Labeling of new DNA was done using a modified protocol described by Ferullo et al. (1). Cells were subsequently pelleted and washed twice with filtered PBS solution, and incubated at room temperature for 30 min in Click-iT reaction mixture (Invitrogen) containing Alexa Fluor 594. Cells were washed twice in PBS solution and spotted on 1% agarose pads for microscopy.

Introduction of the C. crescentus Partitioning System into E. coli. To introduce the C. crescentus partitioning system into the E. coli chromosome, the 0.6-kb fragment in gidA promoter region containing the parS cluster (2) from WT C. crescentus CB15N was cloned into a plasmid and subsequently inserted into E. coli yieM locus, which is 2.3 kb from E. coli oriC, using λ-red recombination. PopZ-YFP was expressed from a plasmid with pBR322 origin under the control of arabinose promoter (pGB268) (3). CFP-ParB (cloned from pGB331) (2) and ParA was expressed from a p15A plasmid under the control of rhamnose promoter. L-arabinose 0.2% and/or 0.02% L-rhamnose was added for induction.


**Table 1.**

<table>
<thead>
<tr>
<th></th>
<th>Generation time (min)</th>
<th>Origin / cell</th>
<th>(C+D)</th>
<th>Initiation</th>
</tr>
</thead>
<tbody>
<tr>
<td>oriC</td>
<td>99(±4)</td>
<td>1.75</td>
<td>79(±4)</td>
<td>21(±4)</td>
</tr>
<tr>
<td>oriC-oriZ</td>
<td>103(±5)</td>
<td>1.7</td>
<td>82(±5)</td>
<td>23(±5)</td>
</tr>
<tr>
<td>oriZ</td>
<td>110(±6)</td>
<td>1.75</td>
<td>90(±5)</td>
<td>21(±5)</td>
</tr>
</tbody>
</table>

Data from flow cytometry experiments, origin/cell=[(1x1)+(2x2)]/100, where r1 and r2 are the ratio of cells containing one or two genome equivalents after rifampicin-cephalexin run-out. For oriC-oriZ, origin pairs are counted.

Calculated using origin/cell = 2^{C+D}/τ, C and D period, and τ the generation time.

Calculated from Initiation = -(C+D).

**Fig. S1.** (A) Cell length distribution measured by flow cytometry of steady-state and late stationary phase cells grown in M9 minimal medium with 0.2% glycerol. oriC, oriC-oriZ, and oriZ strains display very similar cell length distribution in both conditions. (B) DNA content distribution in exponential growth and after rifampicin/cephalexin treatment (Materials and Methods). The stationary-phase pattern is used to calibrate the DNA content corresponding to one and two genome equivalents. The table underneath summarizes the calculations of cell cycle features. (C) Cell volume and DNA content at initiation in oriC strain calculated by using microscopy and flow cytometry data, as well as the Helmstetter–Cooper model (http://simon.bio.uva.nl/cellcycle/index.html).

A. Time-lapse analysis of ori1 and R2 chromosome loci

oriC (n=10)  oriC-oriZ (n=10)  oriZ (n=10)

oriC separated at f8=27.5 (±2.5) min
R2 separated at f8=27.5 (±2.5) min
ori1 / R2 separate at f8-9=40 (±5) min
ori1 separated at f8=27.5 (±2.5) min
R2 separate at f8-9=40 (±5) min
ori1 separate at f8-10=45 (±5) min

B. Replisome quantification in steady state growth

C. Sites of nucleotide incorporation after initiation

D. Snapshots of ori1 and R2 localization after replication

E. Snapshots of ori1 and R2 loci localization in steady-state growth

Fig. S2. (A) Time-lapse analysis of relative positioning of replisomes, ori1 and R2, in oriC, oriC-oriZ, and oriZ strains (5 min per frame). The traces and variances were generated by using an automated particle tracking methodology (Materials and Methods). Cell poles (black line), ori1 (blue line), R2 (green line) are monitored. A representative time-lapse image set is shown below, with indicated times in minutes. If sister loci separation is observed frame X, loci separation could have occurred between X – 1 and X, leading to a 5-min uncertainty. The average timing of separation reported in Fig. S3 is then considered to be exactly between X and X – 1, with a ±2.5 min uncertainty. (B) Left histogram shows the number of DnaN foci per cell in steady state growth, in oriC, oriC-oriZ, and oriZ. Right histogram shows the number of DnaN foci per cell among cells smaller than 2.5 μm in length. Micrographs show exponentially growing cells, with simultaneous localization of DnaN-Ypet (green), ori1 (red), and R2 (blue). (C) Fluorescent labeling of newly replicated DNA. oriC and oriC-oriZ strains were

Legend continued on following page
synchronized by using dnaC2 allele at restrictive temperature, and allowed for initiation at permissive temperature in the presence of nucleotide analogue EdU, which was later fluorescently labeled by using “click” chemistry (SI Materials and Methods). The proportion of cells with one to four foci is shown for oriC and oriC-oriZ strains at 8 and 15 min after release (Left). The data were obtained by counting more than 400 cells for each strain. Representative micrographs for both strains at 15 min after release are shown (Right). (Scale bar, 2 μm.) (D) Histogram of the relative positioning pattern of ori1 and R2 after replisome disappearance, observed for exponentially growing oriC, oriC-oriZ, and oriZ strains. (E) Snapshot analysis of ori1 and R2 loci positioning in exponential growth. The distance between cell pole and foci was measured for 300 cells and reported on the dot plots for ori1 (red) and R2 (blue). The graph on the right shows the average number of ori1 and R2 as a function of the cell size and indicates the relative separation of sister ori1 and R2.

Fig. S3. Attempts to tether the E. coli origin region to a cell pole by using the C. crescentus PopZ-ParAB-parS system. (A) oriC cells were transformed with two compatible plasmids that express arabinose-controllable PopZ-YFP and rhamnose-controllable CFP-ParB and ParA (SI Materials and Methods). Top: Arabinose was added to induce PopZ-YFP (green). Middle: Rhamnose was added to induce CFP-ParB (red) and ParA. Bottom: Arabinose and rhamnose were added for induction. (B) AB1157 carrying parS sites at yieM (2.3 kb from oriC) was transformed with the two plasmids. Rhamnose was added to induce the expression of CFP-ParB (red) and ParA. DAPI stain is in blue. (C) AB1157 carrying parS sites at yieM, ori1 (lacO), and chromosomal LacI-mCherry (red) constitutively expressed from the dnaA promoter was transformed with the two plasmids. Top: Rhamnose was added to induce the expression of CFP-ParB (green) and ParA. Middle: Arabinose was added to induce PopZ-YFP (green). Bottom: Arabinose and rhamnose were added to induce PopZ-YFP (blue) and CFP-ParB (green) and ParA. Yellow arrows show the colocalization of ori1 (lacO; LacI-mCherry), PopZ-YFP, and CFP-ParB. The data show that ParB associates with PopZ at poles when parS sites are absent. In the presence of parS, 76% of cells (n = 879) had ParB foci bound to parS at the normal oriC position and PopZ foci at the poles by free ParB unassociated with parS. The remaining 24% of cells had additionally recruited PopZ to ParB-bound parS sites at the normal oriC position. Therefore, polar PopZ is unable to tether the oriC region to poles through interactions with ParB-parS.