Independent Segregation of the Two Arms of the *Escherichia coli* ori Region Requires neither RNA Synthesis nor MreB Dynamics

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The mechanism of *Escherichia coli* chromosome segregation remains elusive. We present results on the simultaneous tracking of segregation of multiple loci in the ori region of the chromosome in cells growing under conditions in which a single round of replication is initiated and completed in the same generation. Loci segregated as expected for progressive replication-segregation from oriC, with markers placed symmetrically on either side of oriC segregating to opposite cell halves at the same time, showing that sister locus cohesion in the origin region is local rather than extensive. We were unable to observe any influence on segregation of the proposed centromeric site, migS, or indeed any other potential cis-acting element on either replication arm (replichore) in the AB1157 genetic background. Site-specific inhibition of replication close to oriC on one replichore did not prevent segregation of loci on the other replichore. Inhibition of RNA synthesis and inhibition of the dynamic polymerization of the actin homolog MreB did not affect ori and bulk chromosome segregation.

The chromosome of the extensively studied bacterium *Escherichia coli* undergoes simultaneous replication and segregation and has no apparent mitotic apparatus for chromosome segregation, a situation very different from that of eukaryotes, where replication and segregation occur in temporally separate periods of the cell cycle. An unsolved mystery of the bacterial cell cycle is how chromosome segregation takes place. Several mechanisms have been proposed to drive the segregation of origin and bulk DNA after replication. In one model, cell elongation is proposed to be a crucial factor, in which the two newly replicated origins are attached to the inner membrane and separated by cell growth between them along the long axis of the cell (25). However, it is now clear that elongation occurs throughout the cell and the movement of the origins is much faster than the rate of cell elongation, indicating that cell elongation alone is not responsible for segregation (55, 60).

Active partitioning systems were first found in low-copy-number plasmids, where they are required for stable inheritance by distributing the daughter plasmids to both daughter cells (reviewed in reference 14). These systems fall into two families; one uses the ParM actin and its associated protein and binding sites to drive newly replicated sister plasmids apart during cycles of actin polymerization and depolymerization (4, 19). The second parABS family is less well understood mechanistically, although ATP hydrolysis-dependent cycles of ParA movement appear to play a key role in the segregation process (48).

Later, it was found that many bacterial chromosomes also utilize parABS systems for their segregation, for example, *Bacillus subtilis* (23, 37), *Caulobacter crescentus* (41), and both chromosomes of *Vibrio cholerae* (22). The typical chromosomal par locus consists of two genes, parA and parB (soj and spo0J in *B. subtilis*), and a cis-acting parS DNA element. ParB is a DNA-binding protein that specifically recognizes parS and subsequently spreads along the DNA to form a nucleoprotein complex (7, 37, 42). ParA is an ATPase that binds ParB and is proposed to direct the ParB/parS complex to the poles (18). These partitioning systems serve to facilitate chromosome segregation but are often not essential, for example, in *B. subtilis*, *Streptomyces coelicolor*, and *Pseudomonas putida* and for *V. cholerae* chromosome I (18, 23, 30, 35).

In contrast, these systems are essential for viability in *C. crescentus* (41, 54) and for segregation of chromosome II in *V. cholerae* (63). The latter requirement may be due to the fact that chromosome II has many properties of a large plasmid and its Par proteins are more closely related to plasmid-encoded ones than to those encoded on chromosomes (22). In *C. crescentus*, the par system may be essential only indirectly, as it is used for proper localization of the cell division machinery through at least two other proteins, PopZ (6, 13) and MipZ (53). PopZ captures the parB ori complex and subsequently anchors it at opposite cell poles (6, 13). This results in the FtsZ polymerization inhibitor MipZ, which also forms a complex with ParB, to localize to the poles. High concentrations of MipZ at the poles and low concentrations at mid-cell restrict FtsZ ring formation to mid-cell for proper cell division (53).

In a similar indirect manner, Spo0J (ParB) in *B. subtilis* was recently demonstrated to recruit structural maintenance of chromosome (SMC) complexes to the parS sites in the origin region, where these complexes are proposed to organize the origin region and promote efficient chromosome segregation.
Furthermore, in sporulating *B. subtilis*, a different mechanism is used. RacA protein binds to a number of sites within ~200 kb of the origin and then attaches the chromosome to the cell pole in the forespore compartment in a process that also requires SotJ and the polar localized cell division protein DivIVA (5, 62). This process prevents the formation of DNA-free forespores.

*E. coli* and some of its gammaproteobacterial relatives do not encode any obvious *paraAB* system for chromosome segregation (39). It is interesting that these same bacteria have a divergent functional analog to SMC complexes made up of MucB, MucE, and MucF (50) and use SeqA to modulate the initiation of replication (reviewed in reference 56). An *E. coli* 25-bp cis-acting site (*migS*) capable of facilitating bipolar segregation of the origin region has also been described (16, 64). However, in the same studies, deletion of *migS* was shown to have little effect on overall segregation, suggesting that the sequence is not important or is functionally redundant.

A body of experimental evidence has indicated that the chromosome loci segregate sequentially after replication, with a relatively short period of cohesion (36, 43, 47, 57). These data, in part, provided support for an “extrusion-capture” model for chromosome segregation in which a DNA replication factory located at a fixed cellular position pulls in the DNA to replicate and then expels the newly replicated sisters outward (34). However, the demonstration that sister replicosomes track independently along the DNA in slow-growing *E. coli* argues against this model (47). Other observations have led to the suggestion that the organization and properties of the *E. coli* chromosome lead to “segmented” chromosome segregation in which extensive regions of the chromosome segregate together (2, 15).

In a different model, both transcription itself and the coordinated transcription of membrane proteins and their insertion into the membrane (“transertion”) have been proposed as processes that can drive chromosome segregation (12, 45, 49, 61). Nevertheless, these proposals have not been tested rigorously by experiments.

Additionally, it has also been proposed that the highly conserved actin-like cytoskeletal element MreB may play a key role in at least origin segregation (20, 31, 32, 51). MreB polymerizes to form spiral-like filaments that span the inner surface of the cytoplasmic membrane and is responsible for maintaining the cell shape (9, 17, 26). Recent studies have taken advantage of a small molecule, A22 (24), which rapidly disrupts MreB localization *in vivo* by directly binding to its ATPase pocket, inducing a state with low affinity for polymerization (3).

Several studies have demonstrated that inhibition of MreB polymerization does not perturb initiation and progression of DNA synthesis but does apparently block segregation of newly replicated origins, but not bulk DNA (20, 31). Nevertheless, since MreB can serve as a cytoskeletal track for other proteins, its apparent role in origin segregation could be indirect, as supported by a reexamination of the role of MreB in *E. coli* DNA segregation (29).

Results

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** *E. coli* AB1157 strains containing lacO and tetO arrays were constructed as described previously (33, 57, 58). In brief, a lacO or tetO operator array on a plasmid (33) was PCR amplified using primers that provided 45 to 50 nucleotides (nt) of homology on each side of the target insertion locus. The PCR product was transformed to *E. coli* and integrated into the chromosome using λ red recombination (11). To construct the tandem lacO-tetO array, a tetO array (120 copies) was liberated from pLAU40 using NheI/HindIII and ligated to XbaI/HindIII-digested pLAU57, which contains 120 copies of lacO. The resulting plasmid was digested with XbaI/NdeI and ligated with the chloramphenicol resistance gene amplified from pKD3 using primers flanking the NheI/NdeI sites. A tetO-lacO tandem array, followed by a chloramphenicol resistance gene, was constructed using the same method by introducing the lacO array into the plasmid containing the tetO array. The tandem arrays were integrated into the chromosome using λ red recombination (11).

LacC-FP and TetR-YFP (where CFP is cyan fluorescent protein and YFP is yellow fluorescent protein) were expressed constitutively from pW6x (58). Unless otherwise stated, cells were grown at 37°C in M9 minimal medium supplemented with 0.2% glycerol and essential nutrients (58). For exponential growth, 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 40 ng/ml anhydrotrehalose (AT) were added to the medium to reduce repressor binding and allow normal replication and focus visualization. For replication blocks, exponentially growing cells were collected and washed using the same medium lacking AT by centrifugation (8,000 rpm, 1 min, three times) and subcultured in prewarmed AT-free medium. Cells were generally imaged 70 to 100 min after the block was applied, unless otherwise stated. The concentrations of the antibiotics used were 300 μg/ml for rifampin, 10 μg/ml for A22, and 100 μg/ml for ampicillin.

**Fluorescence microscopy.** Cells were grown to an *A* of 0.1 to 0.2 and transferred onto a slide containing 1% agarose in the same medium. Cells were generally imaged 70 to 100 min after the block was applied, unless otherwise stated. The concentrations of the antibiotics used were 300 μg/ml for rifampin, 10 μg/ml for A22, and 100 μg/ml for ampicillin.

**RESULTS**

Segregation patterns of five loci in the *E. coli* ori region. Our previous studies have shown that genetic loci in the terminus region (*ter*), up to 400 kb apart, have a distinct spatial organization, with loci replicated by different forks frequently locating to opposite cell poles during most of the cell cycle (38, 58). This then raises the questions of whether origin regions on either side of oriC behave in the same way during chromosome segregation and whether any part of the origin region segre-
gates first. In order to address these questions, extensive analyses were carried out with markers in the 423-kb region in the origin region, including oriC and migS, the latter a 25-bp sequence 210 kb clockwise from oriC, which was reported to act like an E. coli centromere (Fig. 1A) (16, 64). Cells for all experiments were grown at 37°C in minimal-glycerol medium, which resulted in a generation time of 100 min and with most DNA replication initiations occurring a few minutes after birth and terminating within the same generation (57, 58). Representative snapshot micrographs are shown in Fig. 1B to E, and the primary snapshot data are summarized in Fig. 1G. Time-lapse analyses are summarized in Fig. 1F.

Pairwise combinations of markers ori0 (−2 kb from oriC [−, counterclockwise]), ori1 (−15 kb from oriC), and ori2 (+15 kb from oriC [+ , clockwise]) showed superimposition or partial overlapping of foci, suggesting that the position of any of the three markers broadly describes the behavior of oriC, although ~2% of the cells had the −2 kb sister loci (ori0) separated apparently earlier than sisters of the ±15 kb loci (ori2 and ori1, Fig. 1B, red arrow, and G). Despite being only 13 kb between the two closest ori loci, these loci are apparently resolvable both in time and in space.

Pairwise combinations of an oriC-proximal loci (ori0, ori1, and ori2) with ori-distal loci ori3 (−210 kb from oriC) and ori4 (+213 kb from oriC) showed similar timing for initial separation, with 91 to 95% of the cells having the same number of...
foci for each marker (Fig. 1G). In the 5 to 9% of the cells where there were two separated foci for one marker and only one focus for the other (Fig. 1G, rows highlighted in blue), the majority of the cells had the more oriC-proximal marker separated into two foci, consistent with sequential replication-segregation and the view that cells with a single focus rarely had two spatially nonresolvable foci. Furthermore, combination of ori1 with ori2 or of ori3 with ori4 showed that the timing for replication and separation of loci with the same distance from oriC but on opposite replichores were similar in most cells. Only 4% of ori1-ori2 cells and 5% of ori3-ori4 cells had two foci for one marker and one for the other. The snapshots indicate that sister origins may not always segregate symmetrically from mid-cell to quarter positions (Fig. 1B to E, yellow arrows). For each ori marker, about 10 to 15% of the whole population showed asymmetric positioning (Fig. 1B to E, yellow arrows), rather than the “opposed” asymmetry observed for loci in ter (58), despite there being up to ~400 kb separating the markers visualized in both ori and ter.

Time-lapse analysis was used to assess the interval between the initiation of replication and the segregation of the various ori markers. For this, cells carrying an ori marker and expressing a fluorescent replisome component, YPet-DnaN, were grown on microscopic slides. Images were taken every 5 min, and the appearance of the replisome was defined as time zero. The period of DNA synthesis in these cells was ~68 min (+6 min, n = 41; i.e., ~34 kb · min⁻¹), as judged by the time between replisome appearance and disappearance. The time difference between replisome appearance and visible segregation of the ori marker was recorded. Fifty percent of the cells had two separated foci at 13 min for ori1 and ori2 and at 21 min for ori3 and ori4 (Fig. 1F). The separation of the ori-proximal loci at ~13 min after replication initiation is in agreement with previous estimates of cohesion in the ori region (47). Loci ~200 kb downstream of the ori-proximal loci (ori3 and ori4), which were replicated ~6 min after the ori-proximal loci based on the ~34-kb · min⁻¹ replication rate in these cells, separated ~8 min later, indicating that these loci (ori1, -2, -3, and -4) have similar periods of cohesion. To test if deletion of the proposed centromeric sequence would affect the timing of locus separation, the migS locus was deleted from our AB1157 strain. Cells with or without migS behaved identically for ori separation in our analysis (Fig. 1G).

Our data support the view that segregation of loci in the origin region is sequential and symmetric with respect to each replichore, with loci closer to oriC segregating earlier. We found no evidence for asymmetrically positioned cis-acting sites during DNA segregation. Whatever the global domain structure of the ori region, it does not preclude the spatial separation and independent segregation of loci.

Espeli and colleagues (15) have reported rather different conclusions using E. coli MG1655. Although the focus of this study was the dynamic behavior of different genetic loci, using the ParB-parS labeling system, the authors inferred that ori region loci segregated together some substantive time (up to 30 min) after replication, with only subsequently replicated loci in apparent nonstructured regions showing shorter cohesion and sequential replication-segregation. We do not fully understand the reasons for these differences. However, Espeli and colleagues used a richer growth medium (minimal glucose, Casamino Acids) and a lower growth temperature (25°C). With a doubling time of ~120 min, these cells initiated and completed replication in different generations, with synchronous initiation at two origins occurring around 54 min after birth. These cells also had a very long period (~100 min) between the completion of replication and cell division (D period).

Site-specific replication fork blockage does not interfere with replication and segregation of loci on the opposite replichore. We have previously shown that tight binding of fluorescent fusions of either TetR or LacI to arrays of their cognate operators can be achieved when they are expressed in the absence of their inducers (AT and IPTG, respectively) and results in replication blockage at the array (46). Furthermore, replication restarts rapidly upon relief of these tight repressor-binding events. In order to examine the consequence of replication blockage on one side of oriC to segregation of other ori loci, we analyzed a variety of genetic loci after site-specific replication blockage.

In the first experiment, replication was blocked at ori1 (tetO) using TetR-YFP (constitutively expressed from a high-copy-number plasmid, pWX6) by removing AT from the medium. Seventy minutes after the removal of AT, the proportion of cells with one ori1 focus increased from ~25% to 90% (Fig. 2A, green marker). Importantly, loci downstream of the block,
but not close to the terminus region, never duplicated in the 2 to 4 h following the block. Equivalent results were observed with other blocks in ori. An example is shown in Fig. 2C, where a similar block was induced at ori2 (tetO) by growing cells in liquid medium without AT for 100 min. These cells were then transferred to an agarose slide containing the same medium (without AT), and images were captured every 3 min. The locus ~200 kb downstream, ori4 (lacO), never duplicated in the course of the experiment. These findings imply that tight TetR-YFP binding blocked replication and not segregation of the ori2 (tetO) locus and that the marker downstream was not replicated during the course of the experiment by the clockwise fork (because of replication blockage at the upstream tetO array), by the counterclockwise fork (because of ter sites), or because of potential replication barriers created by sequence skew or head-on transcription collisions.

When AT was reintroduced to release the replication block, within 5 min, 66% of the cells had the tetO array segregated (data not shown), and after 10 min, this proportion increased to 81% of the cells (Fig. 2B, green marker). This is consistent with rapid replication restart and the immediate segregation of loci after replication. This contrasts with the general ~15-min cohesion period of newly replicated sister loci before their visible spatial segregation (see above; 47). The period of cohesion is modulated by the activity of topoisomerase IV (TopoIV), which removes the precatenanes that form between newly replicated DNAs (59). We propose that when replication is blocked by tightly bound repressors, there is sufficient time in the 70-min incubation period before release of the replication block for TopoIV to remove precatenanes so that, once replication resumes, segregation of newly replicated sister loci occurs immediately.

We next examined the consequence of blocking replication at ori1 on the replication-segregation of loci on the other replichore (Fig. 3A). After being blocked at ori1 (tetO; ~15 kb from oriC on the left replichore) for 70 min, cells with one ori1 (tetO) focus increased to ~90% as reported above. When the nonblocked locus being monitored was ori4 (lacO; +210 kb from oriC, +240 kb from the block on the right replichore), after 70 min of ori1 blockage, 33% of the cells had one ori4 focus, 22% had two closely spaced ori4 foci, and more than 30% of the cells had two clearly separated ori4 foci. Similarly, after 70 min of replication blockage at ori1, replication-segregation of R2 (lacO; +1,081 kb, midpoint of the whole 2.3-Mb right replichore) occurred identically to that in cells in which there was no replication block (Fig. 3E and G). These data confirm the independent action of replisomes on sister replichores (47) and show that loci on different replichores not only segregate independently but do so in such a way that each is not influenced by inhibition of the replication-segregation of the other replichore.

The segregation of ori2 (lacO; +15 kb from oriC and 30 kb from ori1) in ori1-blocked cells was also examined (Fig. 3A). After replication blockage for 70 min, the percentage of cells with one ori1 focus increased to 90% as reported above. At the ori2 locus, >50% of the cells had one ori2 focus and 16% of the cells had two ori2 foci very close together. Only 12% of the cells had two well-segregated sister ori2 foci, while 15% had an intermediate separation. Therefore, although blocking replication close to oriC on the left replichore does not prevent replication-segregation of loci on the right replichore, the proximity of ori2 to the blocked ori1 locus (30 kb away) does perturb the visible spatial separation of newly replicated ori2 sisters. This perturbation disappears when loci further along on the right replichore are examined. We assume that these differential effects on segregation are a direct consequence of

![Fig. 3. Effect of the replication block on the segregation of other loci. (A to D) Segregation patterns of the other ori markers when replication was blocked at ori1 (A), ori2 (B), ori3 (C), or ori4 (D). More than 90% of the cells in each culture had only one block focus (green), and these cells were further divided into four classes according to the pattern of the nonblocked (red) locus as illustrated: one red focus, two red foci touching each other, two red foci segregated by less than 20% of the cell length, and two red foci segregated by more than 20% of the cell length. The proportion of each cell type is presented in the histogram. The genetic positions of ori1, -2, -3, and -4 relative to that of oriC are illustrated with the block labeled green. (A) Segregation pattern of ori2 and ori4 with blockage at ori1. (B) Segregation pattern of ori1 and ori3 with blockage at ori2. (C) Segregation pattern of ori1, ori2, and ori4 with blockage at ori3. (D) Segregation pattern of ori2, ori3, and ori4 with blockage at ori4. The block was induced for 70 min in liquid culture. Five hundred to 600 cells of each strain were analyzed. (E) Blockage at ori loci did not perturb the segregation of the later loci on the other replication arm. R2 (lacO, red) segregated normally and achieved asymmetric localization as normal when ori1 (tetO, green) was blocked for 70 min. (F) Blocking of later loci did not perturb the segregation of ori loci. ori1 (lacO, red) segregated normally to quarter positions when R2 (tetO, green) was blocked for 70 min. (G) Segregation of R2 (lacO, red) when ori1 (tetO, green) was blocked in a time-lapse progression. Replication was blocked at ori1 for 60 min in liquid culture, followed by another 30 min on the slide, before the time-lapse progression. An image was taken every 3 min. Shown here is a 6-min time interval.](image-url)
Inhibition of transcription does not prevent origin segregation. (A) Growth curves of dnaC(Ts) mutant cells before and after rifampin (rif; 300 µg/ml) treatment. Cells were grown exponentially at 30°C (blue diamonds). At an $A_{600}$ of ~0.1, part of the culture was shifted to 37°C for 2 h to allow synchronization of replication initiation (green dots). The culture temperature (tm) was then shifted back to 30°C to allow replication initiation, and rifampin was added to half of it (red dots). $A_{600}$ was plotted on a logarithmic scale in arbitrary units (arb.U). (B) ori segregation pattern before and after rifampin treatment in a dnaC(Ts) mutant. Cells were grown exponentially at 30°C. The culture was shifted to 37°C for 2 h for synchronization and then to 30°C for 5 min for initiation of replication. The culture was split in two, and rifampin was added to one of them. Samples for microscopy were taken at each temperature shift, at rifampin addition, and at 35 and 70 min after the temperature shift back to 30°C. The proportions of cells with one ori1 (lacO) focus, two foci touching each other, two foci segregated apart, three foci, four foci, and more than four foci are presented in the histogram. More than 500 cells were analyzed at each time point. (C) Time-lapse progression of dnaC(Ts) mutant cells with rifampin treatment. Cells were growing as previously described. After 2 h at 37°C, cells were shifted back to 30°C for 5 min to allow initiation of replication without changing much of the segregation pattern (compare column 3 to column 2 in panel B). Rifampin was then added to the culture for 10 min of incubation before cells were mounted on an agarose slide with medium and rifampin and visualized by time-lapse photography. ori1 (lacO) is shown in green. An image was taken every 10 min.

Inhibition of RNA polymerase does not affect origin segregation. As both transcription and the insertion of newly transcribed-translated proteins into membrane (transertion) have been implicated as mechanisms contributing to bacterial chromosome segregation (12, 31, 45, 49, 61), we wished to test the consequence of inhibiting transcription (and thereby ongoing transertion) on segregation of ori loci. To do so, we synchronized cells for DNA synthesis using dnaC(Ts) mutation (40) and treated them with rifampin (300 µg/ml) to block transcription. dnaC(Ts) mutant cells were grown exponentially at 30°C and then shifted to the nonpermissive temperature (37°C) at an $A_{600}$ of ~0.1 to block replication initiation but allow completion of ongoing rounds of DNA synthesis. After 2 h, the cells were shifted back to 30°C for 5 min to allow initiation of DNA synthesis (47). The culture was subsequently split in two with rifampin added to one of them. The $A_{600}$ of the rifampin-treated culture stopped increasing immediately after treatment, confirming that rifampin inhibited transcription effectively (Fig. 4A). Origin segregation was examined by snapshot fluorescence microscopy of cells with the ori1 (lacO) marker (Fig. 4B). Following inhibition of replication initiation (2 h at 37°C), most of the cells (77%) had a single ori1 focus, as expected. Cells with two foci were likely to be ones that were blocked for initiation but had not divided. After 5 min at the permissive temperature, during which replication initiation can occur, 73% of the cells retained a single focus. Although rep-
llication initiation occurs efficiently under these conditions, most of the newly replicated loci have not segregated (47, 59). By 70 min after replication initiation, the rifampin-treated and rifampin-free cultures showed essentially identical distributions of foci, with >80% of the cells containing two or more ori<sub>1</sub> foci. Nevertheless, 35 min after replication initiation, only 65% of the rifampin-treated cells had two or more ori<sub>1</sub> foci, compared to >80% of the cells in the nontreated control. Therefore, inhibition of transcription appeared to cause a slight delay of the replication-segregation process upon replication initiation.

In a parallel experiment in which rifampin was added 5 min before a shift back to the permissive temperature to allow replication initiation, >80% of the cells contained two or more foci at 70 min at the permissive temperature, while 56% of the cells contained two or more ori<sub>1</sub> foci at 35 min (data not shown). This result implies that inhibition of transcription does not prevent ori<sub>1</sub> locus segregation but may delay replication initiation. Nevertheless, essentially all of the cells were able to initiate replication in a dnaC(Ts) strain shifted to the permissive temperature and to subsequently segregate newly replicated loci under conditions of transcription inhibition.

Time-lapse experiments confirmed the above observations (Fig. 4C). Following synchronization, cells were left at the permissive temperature for 5 min to allow replication initiation and then rifampin was added to the liquid culture for 10 min before the cells were mounted on the agarose slide containing growth medium and rifampin. During an 80-min time-lapse period, 20 of 36 rifampin-treated cells duplicated and separated their ori<sub>1</sub> sister foci more than one-third of a cell length apart, whereas the average cell length increased by only ~3% (compared to ~68% without rifampin treatment). An example is shown in Fig. 4C; the length of the rifampin-treated cell increased from 3.8 to 3.9 μm over 80 min, while the sister ori<sub>1</sub> loci segregated 1.4 μm apart between 20 and 30 min and were maximally 2.3 μm apart. This confirms that inhibition of transcription does not prevent segregation of newly replicated origins and that cell elongation is not necessary to allow ori<sub>1</sub> segregation. Examination of the segregation of R2 and loci within ter after rifampin treatment also showed that segregation of newly replicated copies of these loci was not blocked by inhibition of transcription (data not shown).

**Inhibition of MreB dynamics does not affect origin segregation.** Having shown that neither transcription nor cell growth along the long axis is necessary for ori<sub>1</sub> segregation, we examined the consequences of inhibiting the cytoskeletal protein MreB on ori<sub>1</sub> locus segregation. In *C. crescentus*, A22, an inhibitor of the dynamic polymerization of the bacterial actin-like protein MreB, was reported to completely block the movement of newly replicated origins (20). Similarly, it was reported that after 1 h of A22 treatment, the percentage of *E. coli* cells with two ori<sub>1</sub> foci decreased from 80% to 20%, suggesting that inhibiting MreB dynamics may also block origin segregation in *E. coli* (31). Furthermore, these same authors noted that cells expressing a mutant MreB protein failed to segregate their chromosomes normally (32). However, it has also been reported that A22 does not prevent chromosome segregation in *E. coli* (29). In an attempt to address this inconsistency, we used A22 to study the effect of MreB dynamics on origin segregation in our strain and under our experimental conditions. In all of our experiments, A22 (10 μg/ml) treatment caused cells growing in minimal medium to slowly change their shape from rods to spheres through an egg-shaped intermediate. A strain with a single point mutation in MreB, making it resistant to A22 treatment, did not undergo the shape change (data not shown), showing that A22 did inhibit MreB function in our experiments.

To study the effect of A22 on ori<sub>1</sub> segregation, an exponential culture was split in two and one was treated with A22. Cells growing exponentially were harvested at different time points before and after A22 (10 μg/ml) treatment. The number of ori<sub>1</sub> (lacO) foci was analyzed and plotted. More than 500 cells were analyzed at each time point. (B) A22 does not block origin segregation after synchronization using dnaC(Ts) mutant cells. Cells were grown exponentially at 30°C and then shifted to 37°C for 1 h for synchronization. The culture was then split in two, one with and one without A22 treatment, and grown at 30°C for 1 h. Cells were harvested at each time point, and the ori<sub>1</sub> (lacO) foci in each cell were counted. The proportions of cells with different numbers of origins are shown. More than 500 cells were counted at each time point. (C) Time-lapse progression of cells treated with A22. Exponentially growing cells were treated with A22 for 1 min in liquid culture and subsequently mounted on an agarose slide containing medium and A22. Images were taken at 5 min, 1 h, 2 h, and 3 h after A22 treatment. ori<sub>1</sub> (lacO) was labeled green.
strain was used such that A22 could be added at the time of reinitiation of DNA synthesis, no difference was observed between the A22-treated cells and the nontreated control, again demonstrating no influence of A22 on ori1 segregation (Fig. 5B). Finally, examination of A22-treated cells by time-lapse microscopy confirmed that cells continued to grow and segregate their ori1 sister loci during A22 treatment (Fig. 5C).

Our data therefore support the view that inhibition of MreB dynamics does not prevent segregation of the origin region. Furthermore, we found that L3, R3, and loci in ter were also able to segregate normally after A22 treatment (data not shown), and therefore inhibition of MreB dynamics by A22 does not affect bulk chromosome segregation.

**Dynamic locus behavior in the region of replication blocks.** During initial time-lapse experiments of replication-blocked cells, we noted that with a replication-blocked ori1 locus, the neighboring locus was sometimes seen to split into two closely spaced loci before reverting back to a single focus (Fig. 6A and B, 30-min block in liquid culture, followed by an additional 30 min on the slide before imaging, red arrows), thereby explaining some of the snapshot data (Fig. 3A to D). Furthermore, we occasionally observed a splitting of a blocked locus into two, before reversion to a single focus (Fig. 6B, green arrow). This behavior was not modified after rifampin treatment and was also observed in RecA− cells, indicating that it is not a consequence of transcription/transversion or induced by DNA breakage-recombination (data not shown).

To gain insight into these observations, we constructed hybrid arrays in which 120 copies of tetO (4.1 kb) and 120 copies of lacO (4.3 kb) are immediately adjacent to each other. This tandem array hybrid was inserted at the ori1 locus in both orientations, so that clockwise replication forks could encounter either the lacO or the tetO array first. Therefore, when replication was blocked at tetO, the behavior of lacO when either upstream or downstream of the block could be observed in time-lapse experiments.

When lacO was downstream of the block, only a single lacO focus, representing the unreplicated locus, was ever observed (Fig. 6C, 70-min block in liquid, followed by an additional 30 min on the slide before imaging; see movie S1 in the supplemental material). At most time points (93% in 25 time progressions, each of 14 time points), the lacO and tetO foci were superimposed.

When lacO was immediately upstream of the block, we observed frequent splitting and refusion of lacO foci (Fig. 6D, 70-min block in liquid, followed by an additional 30 min on the slide before imaging; see movie S2 in the supplemental material), indicating that a replication fork Y structure with two newly replicated copies of lacO adjacent to at least a partly unreplicated tetO locus allows spatial resolution of the sister lacO loci. Note that in these time-lapse progressions, as in those in Fig. 6C and D, the blocked locus exhibits an occasional splitting in two and refusion, indicating that the blocked locus has undergone at least partial replication, allowing some separation of the newly replicated tetO sisters. Analysis of 22 time-lapse progressions showed that the time during which the upstream lacO locus is split in two is about three times that of the blocked tetO locus.

The ability to spatially separate newly replicated sister lacO foci immediately adjacent to a blocked tetO locus was unexpected and shows that sister loci that are close and physically linked can be spatially separated. The length of an uncompact ed 4.3-kb lacO locus is 1.46 μm; we do not know the conformation of such a locus in vivo and do not know how much of an array has to be bound by fluorescent repressors to give a focus. We would not expect sister copies of such a locus immediately behind a fork to become supercoiled because of the free DNA ends at the fork. Nevertheless, upon repressor binding, we observe a sharp focus indistinguishable from foci distant from replication forks. In Fig. 6D, the separated sister lacO foci are frequently ≥1 μm apart, with a smeary TetR-YFP trail between them. The 18- to 24-min time points in Fig. 6D are shown in Fig. 6E with the LacI-CFP (red) and TetR-YFP (green) channels placed side by side, along with a schematic that illustrates what we believe is the explanation for our observations. Assuming that the replication block is contained within the 4.1-kb tetO array, the centers of the upstream 4.3-kb sister lacO arrays can have a maximal spatial separation of 4.3 kb to <1.25 kb (1.46 to 4.25 μm uncondensed), depending on whether the fork is blocked at the beginning of the tetO array or toward its end. The outer extremities of the newly replicated arrays could be up to 16.8 kb apart. About three kilobases of an uncondensed newly replicated locus bound by the repressor at its compacted outer extremity (Fig. 6E, schematic) would allow the type of spatial separation that we observed, with the uncondensed array sequence either lacking fluorescence or with a smear of fluorescence instead of a sharp focus.

Another explanation for the observed splitting of the lacO sisters when the downstream tetO locus is replication blocked is the arrival of a new round of replication at the blocked locus that generates a double-strand break at the blocked fork. This physical unlinking would allow separation of the lacO sisters as observed. Nevertheless, we do not favor this explanation since this behavior is observed in cells blocked for a period of only one generation (Fig. 6C and D) or less (Fig. 6A and B) before image capture commences. Because there are no overlapping rounds of replication under our growth conditions, most of the blocked forks would not be encountered by other replication forks from a new round of replication within the time course of the experiment. Furthermore, the same behavior is observed in RecA− cells, in which we would not expect to see the refusion of the foci if the splitting were caused by double-strand breaks when new replication forks run into a blocked fork. We note that in these experiments, there is sufficient time for any pretreatment present in the vicinity of the newly replicated sister loci to have been removed by TopoIV, which in turn may allow enough separation of the loci to explain the detectable splitting. These results provide new information on the spatial resolution of newly replicated loci using conventional epifluorescence microscopy and suggests dynamic behavior of newly replicated DNA in the region of a blocked fork.

**DISCUSSION**

The work presented here provides no support for the idea that transcription/transversion, MreB dynamics, or specific cis-acting DNA sequences play a key role in E. coli chromosome segregation. In cells with a single round of replication initiated and terminated in the same generation, segregation of the newly replicated sister loci of the ori region on different repli-
A. ori1 (block), ori2 (no block), (30 min block in liquid+30 min on slide), 10 min/image

B. ori1 (block), ori2 (no block), (30 min block in liquid+30 min on slide), 10 min/image

C. ori2 tetO (block) - lacO, (70 min block in liquid+30 min on slide), 3 min/image

D. ori2 lacO - tetO (block), (70 min block in liquid+30 min on slide), 3 min/image

E. lacO - tetO (block), offset, reshow of 18 min-24 min from D.

FIG. 6. Dynamics of replication block by TetR binding to tetO in time-lapse progressions (left) and their plots (right). (A and B) Dynamics of ori1 and ori2 during replication block at ori1 (tetO). To induce a block, cells were grown in liquid culture for 30 min without AT, followed by another 30 min without AT on the slide. An image was taken every 10 min. Red and green arrows show the splitting and refusion of the nonblocked locus and the blocked locus, respectively. (C and D) Dynamic behavior of tandem tetO-lacO or lacO-tetO arrays at ori2. In panel C, the lacO array (labeled red) is downstream of the tetO (green) block. In panel D, the lacO array (labeled red) is upstream of the tetO (green) block. Red and green arrows show the splitting and refusion of the nonblocked locus and the blocked locus, respectively. The block was induced for 70 min in liquid culture, followed by an additional 30 min on the slide before image capture. Images were taken every 3 min. (E) Eighteen- to 24-min time points from panel D with the TetR-YFP (green) and LacI-CFP (red) channels placed side by side for clarity. The schematic on the right illustrates the explanation. Condensed lacO and tetO arrays are shown as red and green helices. Replication block occurs within the tetO array, which allows replication of the upstream lacO array. At 18 min, both the lacO and tetO arrays were condensed, giving one red focus and one green focus overlapping due to the limitation of spatial resolution. At 21 min, 2 to 3 kb of DNA in one of the replicated lacO arrays was relaxed, or stretched by the upstream DNA regions (not illustrated), so that the beginning of that lacO array was separated far enough to give a separate focus. At 24 min, some of the replicated tetO array was condensed with the upstream lacO array, leaving a smear of TetR-YFP (green) fluorescence labeling the uncondensed region. Note that YFP is significantly brighter than CFP owing to the properties of the different fluorophores, which is why the smear of the CFP (labeled red) channel is not visible or is less clear.
chores is sequential, independent, and apparently symmetrical. Blocking of replication in one replichir does not prevent segregation of loci on the other.

It seems plausible that spontaneous chromosome segregation by entropic disentanglement of the chromosome-related (7, 28) may provide the essence of the segregation mechanism. Therefore, the key to efficient and faithful segregation is likely to reside in chromosome organization itself and the processes that drive this organization, as well as independent replication by spatially separated replisomes tracking along the DNA and the subsequent decatenation by TopoIV. Consistent with this view, aberrant chromosome organization as a consequence of absence of functional SMC complexes (MukB-EF) leads to an altered pattern of replication-segregation and to failures in chromosome segregation (8, 10, 44). It seems to us with this view, aberrant chromosome organization as a consequence of absence of functional SMC complexes (MukB-EF) leads to an altered pattern of replication-segregation and to failures in chromosome segregation (8, 10, 44). It seems to us

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