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Gyrase Inhibitors and Thymine Starvation Disrupt the Normal Pattern of Plasmid RK2 Localization in *Escherichia coli*

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Multicopy plasmids in *Escherichia coli* are not randomly distributed throughout the cell but exist as defined clusters that are localized at the mid-cell, or at the 1/4 and 3/4 cell length positions. To explore the factors that contribute to plasmid clustering and localization, *E. coli* cells carrying a plasmid RK2 derivative that can be tagged with a green fluorescent protein-LacI fusion protein were subjected to various conditions that interfere with plasmid superhelicity and/or DNA replication. The various treatments included thymine starvation and the addition of the gyrase inhibitors nalidixic acid and novobiocin. In each case, localization of plasmid clusters at the preferred positions was disrupted but the plasmids remained in clusters, suggesting that normal plasmid superhelicity and DNA synthesis in elongating cells are not required for the clustering of individual plasmid molecules. It was also observed that the inhibition of DNA replication by these treatments produced filaments in which the plasmid clusters were confined to one or two nucleoid bodies, which were located near the midline of the filament and were not evenly spaced throughout the filament, as is found in cells treated with cephalexin. Finally, the enhanced yellow fluorescent protein-RarA fusion protein was used to localize the replication complex in individual *E. coli* cells. Novobiocin and nalidixic acid treatment both resulted in rapid loss of RarA foci. Under these conditions the RK2 plasmid clusters were not disassembled, suggesting that a completely intact replication complex is not required for plasmid clustering.

The development of methods for tagging bacterial plasmids with specific fluorescent fusion proteins or by fluorescence in situ hybridization has greatly facilitated the microscopic analysis of the location and movement of plasmids in a bacterial cell during growth and division. Contrary to the generally accepted view that plasmids randomly diffuse throughout the cell, it has now been shown that the low-copy-number plasmids F (16, 18, 49), P1 (16, 45), and R1 (31, 63) and the multicopy plasmids RK2 and pUC (4, 25, 53, 54) are not randomly distributed throughout out an *Escherichia coli* cell but are present as clusters at preferred locations. Using differentially labeled probes and fluorescence in situ hybridization analysis, it has been shown that except for plasmid R1, which is located at mid-cell or at the cell poles (31), clusters of plasmids F, P1, RK2, and pUC generally are located at the mid-cell position in newborn *E. coli* cells and at the 1/4 and 3/4 positions in larger cells (16, 25, 44). For these plasmids it has been shown further that the movement of newly duplicated plasmid clusters from the mid-cell to the quarter-cell positions occurs with relatively rapid kinetics (16, 18, 25, 44, 49).

Much has yet to be learned about cell- or plasmid-specified factors that are responsible for the localization and movement of plasmid clusters. It has been shown for plasmids F (18, 49), P1 (6, 44), R1 (63), R27 (39), and pB171 (5) that the mutation of plasmid-encoded partition loci perturbs the regular pattern of plasmid localization or, in the case of plasmid R1, results in an interference with the separation of plasmid pairs and the movement of plasmids to opposite poles in the cell (31). Interestingly, the ParM component of the R1 partitioning system forms double helical protofilaments with features similar to F-actin (48, 61). It has been proposed that ParM moves newly duplicated plasmid R1 molecules by an actin-like insertional polymerization mechanism (15).

Virtually nothing is known regarding the factors that are responsible for the clustering of individual plasmid molecules. One model that has been considered is that the clusters of individual plasmid molecules are tethered to the cellular replisome (53). The finding that DNA replication occurs in stationary factories in the regions of the mid-cell and quarter-cell positions in *Bacillus subtilis* (41) and in *E. coli* (36) at least raises the possibility that plasmid duplication occurs at the mid-cell position and that after duplication the replication complexes with the attached plasmid clusters segregate to quarter-cell positions prior to the next round of cell division. Using a temperature-sensitive replication initiation protein for the F plasmid, evidence has been obtained in support of duplication of this plasmid at the mid-cell immediately followed by movement of each of the duplicated plasmids to their respective quarter-cell positions (51). Consistent with the notion that DNA replication proteins play a role in the segregation of plasmids, or plasmid clusters, to new fixed positions is the report of Miller and Cohen (46) that DNA replication proteins contribute to plasmid pSC101 segregation in addition to their role in replication of this plasmid. It has been argued, however, from studies with derivatives of plasmid pSC101 that are temperature sensitive for replication that declustering of plasmids can occur in the absence of plasmid replication (21, 50). It is also clear that while plasmid-encoded partition proteins play a

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role in the localization of plasmid elements in *E. coli*, par$^-$ derivatives of plasmid R1 continue to form clusters, indicating that although the par system contributes to localization of plasmid clusters it is not the binding force for clustering of individual plasmid molecules (64).

A number of different antibacterial agents have been shown to directly or indirectly inhibit DNA replication and/or cell division, but little is known about the effects of these agents on plasmid localization or movement. Cephalexin, which inhibits cell division but not DNA replication, produces filaments with evenly spaced nucleoids (30). Interestingly, fluorescence microscopy analysis of the location of tagged plasmids in cephalexin-treated cells has demonstrated that plasmid clusters are also evenly spaced along the length of the filaments within the nucleoid bodies (25). Nalidixic acid addition rapidly interferes with DNA replication in *E. coli* by causing the covalent attachment of gyrase to the DNA substrate, with the subsequent production of double-strand breaks in the DNA (12, 22, 23, 58). Novobiocin also inhibits DNA gyrase (13) but, unlike nalidixic acid, this inhibition does not result in the covalent attachment of gyrase to DNA (43, 57) and, therefore, there is no extensive formation of double-strand DNA breaks. The addition of either nalidixic acid or novobiocin also results in filament formation.

In this work, we have examined the effect of the addition of nalidixic acid and novobiocin as well as the inhibition of DNA synthesis by thymine starvation on plasmid RK2 cluster formation and localization in *E. coli*. Plasmid RK2 is present at five to eight copies per chromosome (9, 55, 59, 60). These copies form one or several clusters in *E. coli*, *Pseudomonas aeruginosa*, and *Vibrio cholerae*, depending on cell size and growth conditions (25). For this analysis, a plasmid RK2 construct was used that both expresses the green fluorescent protein (GFP)-LacI fusion protein and contains an array of *lacO* operator sequences that serve as a binding site for GFP-LacI (16, 53). In addition, an enhanced yellow fluorescent protein (EYFP)-RarA fusion protein was used to localize DNA replication complexes (38). Evidence has been obtained for an association of the RarA protein with the *E. coli* replication factory (2, 38). Inhibition of DNA synthesis by thymine starvation or the addition of nalidixic acid or novobiocin results in filament formation. In each case the filaments contain extended nucleoids as measured by 4′,6′-diamidino-2-phenylindole (DAPI) staining and a dislocation of plasmid clusters that are contained within the extended nucleoid region. Furthermore, there is a relatively small increase in the number of plasmid foci, suggesting a partial breakdown of plasmid clusters. Interestingly, both novobiocin and nalidixic acid addition results in a rapid loss of RarA foci without a significant increase in RK2-tagged foci, indicating that an intact replication complex is not required for plasmid clustering.

MATERIALS AND METHODS

Strains, media, and growth conditions. Strains MG1655(Seq) (F$^-$ λ$^-$ *ilvG rfb-50 rph-1*; CGSC 7740) or DG76 [F$^-$ *leuB6*(Am) λ$^-$ *thyA47 rpsL153* (strR) *deoC3*] were grown at 30°C in M63 medium (52) prepared with 0.2% glycerol or in Lennox L broth (LB; Sigma, St. Louis, MO). DG76 cultures were supplemented with thymine at 50 μg/ml. Strains containing plasmid pZZ15 were grown in the presence of penicillin (250 μg/ml) and tetracycline (15 μg/ml), while MG1655(pBS8) was grown in the presence of penicillin. Plasmid pZZ15 (53) is an RK2 derivative containing GFP fused to LacI expressed by the pBAD promoter (19) and approximately 256 *lacO* sequence repeats. Plasmid pZZ15 contains the partitioning and conjugal transfer functions of the native plasmid. Upon induction with L-arabinose, GFP-LacI is produced from pZZ15 and specifically binds to the *lacO* repeats to provide a fluorescent tag of the plasmid. Plasmid pBS8 is derived from pBAD24 and expresses an EYFP-RarA fusion protein after L-arabinose induction (38).

Microscopy. To achieve GFP tagging of plasmids, strains were grown overnight at 30°C in LB and then diluted 1:100 into LB or M63 with 0.2% glycerol as a carbon source (time zero). After growth at 30°C to an optical density at 600 nm (OD_{600}) of 0.1 over inoculum, GFP-LacI expression was induced by adding L-arabinose to a final concentration of 0.6% and then, after the appearance of tagged plasmid foci, repressed by adding glucose to a final concentration of 0.6%. Foci typically became visible approximately 1 h after induction with L-arabinose. MG1655(pZZ15) was treated with 15 μg/ml nalidixic acid at 3 h, with 1 mg/ml novobiocin at 4 h, or with 200 μg/ml cephalexin immediately after inoculation. DG76(pZZ15) cells were grown either with or without thymine at 50 μg/ml as a supplement. Samples were harvested at various time points for visualization by fluorescence microscopy.

For tagging of replication complexes, MG1655(pBS8) was grown overnight in LB at 30°C and then diluted 1:100 into 20 ml of M63 with 0.2% glycerol as the carbon source. After reaching an OD_{600} of 0.2 over preinoculated culture, cultures were diluted 1:4 and EYFP-RarA expression was induced by adding L-arabinose (in M63) to a final concentration of 1%. After an additional 3 h of growth, the culture was split into three portions: one portion was untreated and the other two were treated with 15 μg/ml nalidixic acid or 1 mg/ml novobiocin (final concentration).

For visualization by fluorescence microscopy, 8 μl of cells were stained with DAPI (5 μg/ml) and added to poly-lysine-treated slides without fixation. Cells were visualized with a BX60 fluorescence microscope (Olympus), and images were captured with a C-5050 digital camera (Olympus). Cells and foci were measured using public domain NIH Image software (National Institutes of Health; http://rsb.info.nih.gov/nih-image/).

Thymidine incorporation assay. *E. coli* strain MG1655 was grown overnight at 30°C in LB medium supplemented with 50 μg/ml thymidine. After 1:100 dilution with fresh medium, cells were grown at 30°C to an OD_{600} of 0.1 and 100 μCi [^{3}H]thymidine was added to the medium. After an additional 90 min of growth, cells were either untreated or treated by adding at a final concentration of 15 μg/ml nalidixic acid or 1 mg/ml novobiocin to the cultures. At 15- or 30-min intervals, 200-μl samples were removed and incubated for 5 min at 4°C in 5 ml of prechilled 5% trichloroacetic acid with 1 mg/ml cold thymidine to precipitate DNA (8). Samples were then passed through GF/C filters and washed twice with 5 ml of 5% trichloroacetic acid at 4°C, three times with 1 ml of water, and twice with 1 ml of 95% ethanol. Filters were dried and subjected to liquid scintillation counting.

RESULTS

Effects of nalidixic acid, novobiocin, and cephalexin on RK2 localization. Nalidixic acid and novobiocin are known to inhibit *E. coli* gyrase activity (12, 13, 58). As a consequence of gyrase inhibition, there is a rapid inhibition of DNA synthesis (23) and eventually cell division, leading to the formation of filamentous cells. A thymidine incorporation assay was performed to determine the timing and extent of DNA inhibition by nalidixic acid and novobiocin under the conditions of our experiments. As shown in Fig. 1A, there was an apparently immediate inhibition of [^{3}H]thymidine incorporation after the addition of either drug compared to untreated cells.

The RK2 derivative pZZ15 was used to localize this plasmid in MG1655 cells grown in minimal medium. Plasmid pZZ15 encodes an L-arabinose-inducible GFP-LacI fusion protein and a *lacO* binding site cassette. Addition of L-arabinose results in expression of GFP-LacI and specific binding of this fluorescent fusion protein to the plasmid. After approximately 1 h of treatment, nalidixic acid-treated cells began to filament. By 3 hours after treatment, some nalidixic acid-treated cells achieved a length of approximately 10 μm. Some of the elongated cells contained as many as eight plasmid foci, compared with no

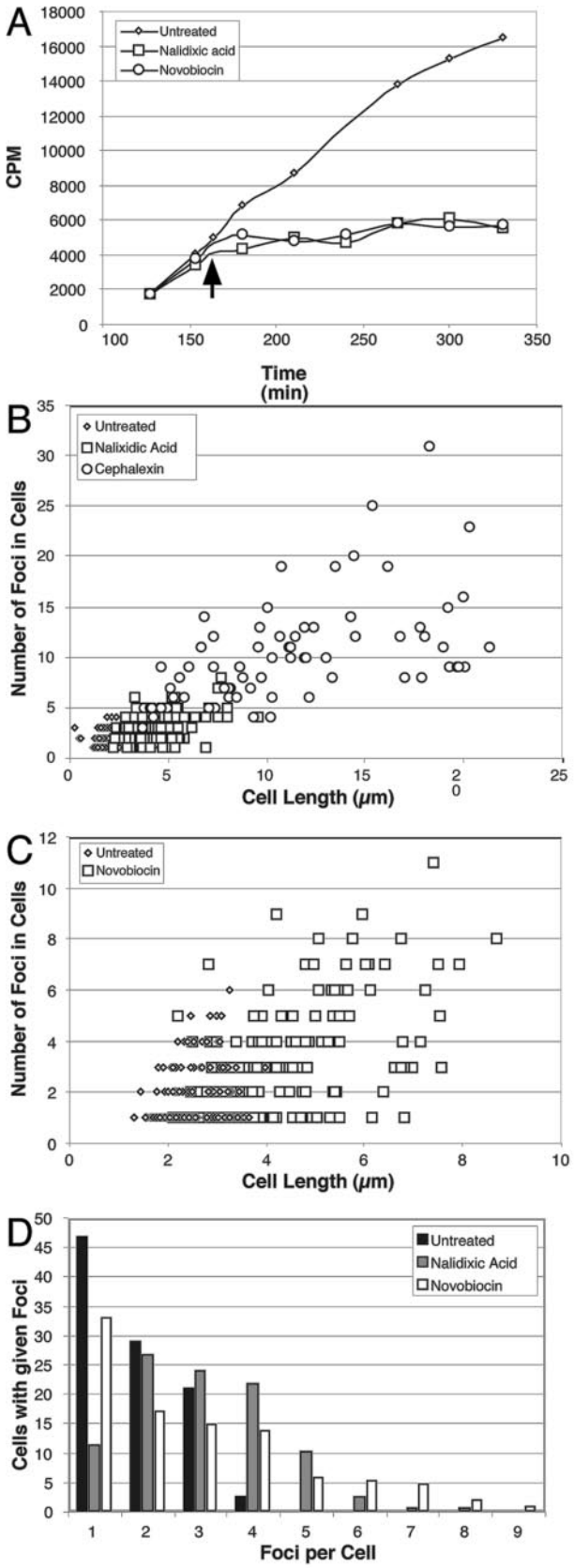

FIG. 1. Treatment of MG1655(pZZ15) cells with nalidixic acid, novobiocin, and cephalexin. A. Thymidine incorporation during growth of MG1655(pZZ15) cells either untreated or treated with nalidixic acid or novobiocin. The black arrow indicates time of addition of inhibitor. B. Foci per cell versus cell length for cells either untreated or treated with nalidixic acid for 3 h or cephalexin for 5 h. Each point represents one cell. A total of 325 cells were counted. C. Foci per cell versus cell length for cells either untreated or treated with novobiocin for 4 h. Each point represents one cell. A total of 325 cells were counted. D. Histogram of cells with a given number of foci. The *y* axis is expressed as the percentage of total cells with a given number of foci. Cells were either untreated or treated with nalidixic acid for 3 h or novobiocin for 4 h. A total of 254 cells were counted. Over 90% of the nalidixic acid-treated cells and 98% of the novobiocin-treated cells that were stained with DAPI contained a single nucleoid; the remaining cells contained two nucleoids. The results presented are from a single experiment for each treatment, but each treatment was carried out in more than one experiment with similar results.

more than five in untreated cells (Fig. 1B and D). This increase in foci number upon treatment with nalidixic acid under conditions of DNA replication inhibition suggests some destabilization of plasmid clusters. However, the possibility that this increase in the number of plasmid foci is due to less-than-complete inhibition of plasmid replication cannot be ruled out.

It was also observed that in cells treated with nalidixic acid, foci were often grouped near the midline of the elongated cell (Fig. 3B and H). In untreated cells with two foci, the foci were located at approximately the 1/4 and 3/4 positions, as expected (Fig. 2A and 3A and G). In nalidixic acid-treated cells with two foci, however, the foci were not well spaced but tended to be centered around the cell midline (Fig. 2B and 3B and H). Of the nalidixic acid-treated cells in our study, 65% were filamentous with a midline grouping of two or more foci. The remaining cells were filamentous with foci dispersed throughout the cell (7%) or were cells of normal length with foci dispersed randomly throughout the cell (28%). Staining of the nucleoid by DAPI revealed that foci were almost always confined to the region of the nucleoid in nalidixic acid-treated cells (Fig. 3E and H).

Novobiocin had a similar effect on the distribution of plasmid foci. Like nalidixic acid-treated cells, many cells were elongated with an approximately twofold increase in the number of foci 4 hours after the addition of novobiocin (Fig. 1C and D and 3K). In addition, as with nalidixic acid treatment, the addition of novobiocin resulted in the grouping of foci near the midline and within the region of the nucleoid as revealed by DAPI staining. After 4 h of treatment 65% of novobiocin-treated cells had one or two foci at locations typical of untreated cells, while 31% demonstrated mid-cell grouping of foci (Fig. 3K, panel i). These effects of drug treatment were even more pronounced after 24 h (Fig. 3L, panel i). There were, however, some differences between nalidixic acid- and novobiocin-treated cells. In addition to cells with midline-grouped foci, 4% of novobiocin-treated cells had foci with a "spaced" grouping 4 h after treatment (Fig. 3K, panel ii). In these cells, foci were predominately in two groups, one on either side of the cell midline. After 24 h of treatment, the number of cells with spaced grouping increased to 14% of the total (Fig. 3L, panel ii).

The localization of plasmid foci after treatment with nalidixic acid or novobiocin is unlike that observed with cephalexin addition. Growth of cephalexin-treated MG1655(pZZ15) cells in either M63 prepared with glycerol (Fig. 3C, F, and I) or LB

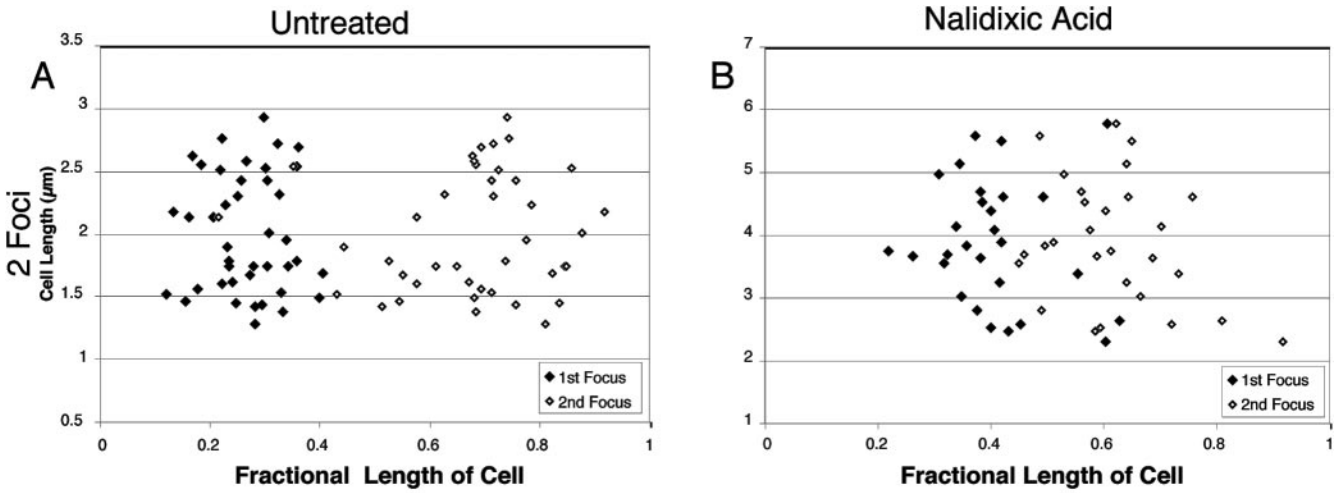

FIG. 2. Nalidixic acid interference with RK2 localization. Position of individual foci in MG1655(pZZ15) cells with two foci (A and B) and untreated (A) or treated with nalidixic acid (B). The *y* axis is the length in micrometers of the host cell, while the *x* axis is the fractional distance of the foci from the cell pole. Each point represents a single focus and is represented by the first focus and second focus from a pole.

medium (Fig. 3J) resulted in filaments with evenly spaced nucleoids with the foci located within the nucleoid bodies. Since cephalexin does not inhibit DNA replication, the filaments contained large numbers of evenly spaced foci (Fig. 1B and 3C and I). This suggests that the mislocalization of plasmid clusters seen with nalidixic acid and novobiocin treatment, and not seen with cephalexin treatment, is directly or indirectly due to the inhibition of DNA replication and not the inhibition of cell division alone. In 35% of cephalexin-treated cells, the filament contained a region with a large number of somewhat

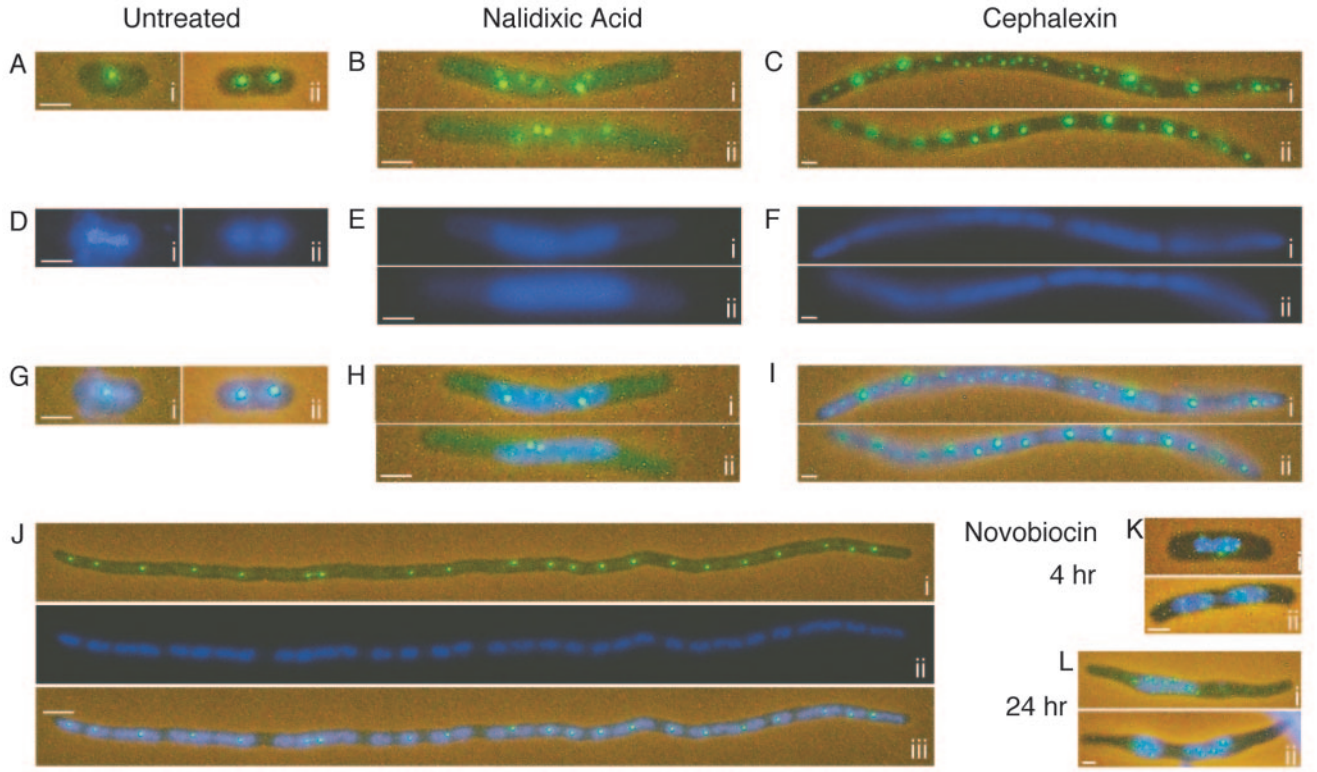

FIG. 3. Effect of inhibitors on the positions of nucleoids and RK2 foci. MG1655(pZZ15) cells were untreated (A, D, and G) or treated with 15 μg/ml nalidixic acid for 3 h (B, E, and H), 200 μg/ml cephalexin for 4 h (C, F, I, and J), or 1 mg/ml novobiocin for 4 (K) or 24 (L) hours. Cells were visualized by fluorescence microscopy, and plasmid foci were visualized by tagging with LacI-GFP (A, B, C, and J, i panels). Chromosomal DNA was stained with DAPI (D, E, F, and J, ii panels). GFP and DAPI images were merged to give the images shown in panels G, H, I, J (panel iii), K, and L.

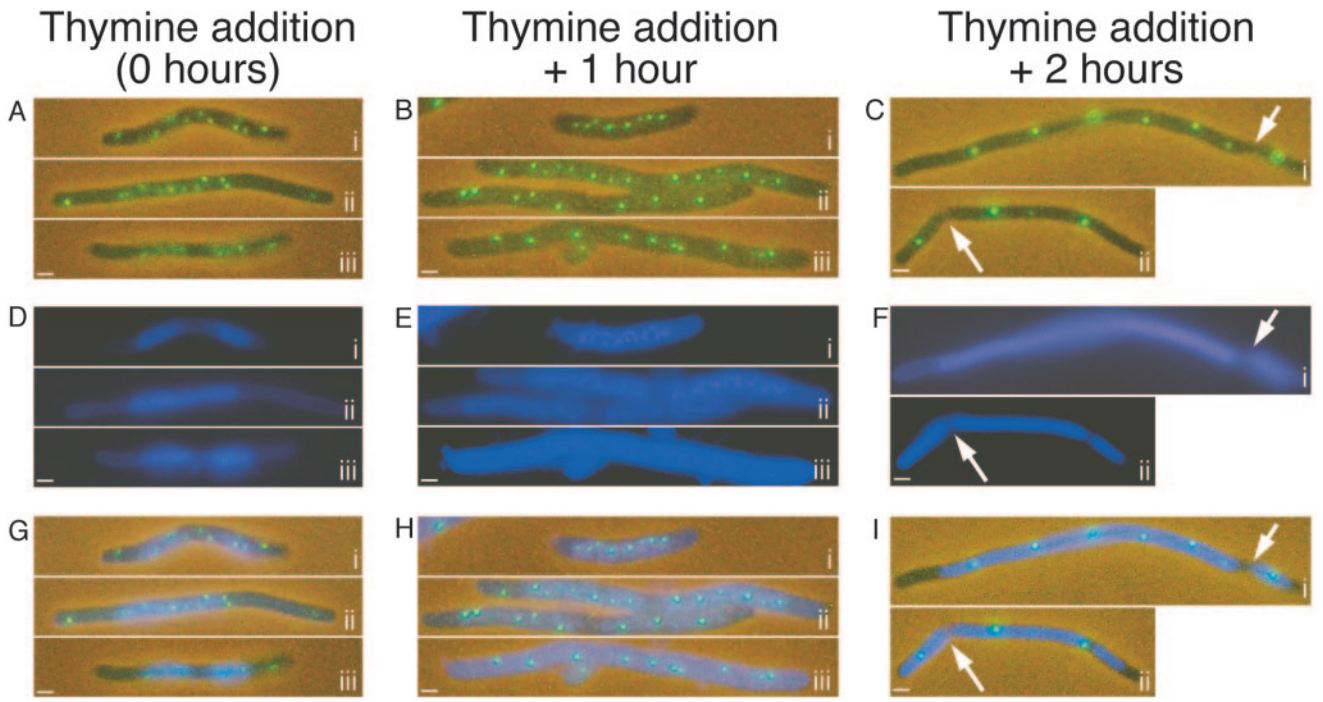

FIG. 4. Plasmid localization after thymine starvation and recovery. DG76(pZZ15) cells were grown without thymine for 6 h (A, D, and G), and then thymine was added to the growth medium. Cells were then grown for an additional 1 h (B, E, and H) or 2 h (C, F, and I). (A, B, and C) Visualization with GFP and bright field; (D, E, and F) staining with DAPI; (G, H, and I) merged GFP, bright field, and DAPI images. White arrows show the positions of cell division septa.

less intense foci (Fig. 3C, panel i, and I, panel i), suggesting declustering of foci in these regions, but these foci were evenly distributed throughout the region and did not show the localization pattern characteristic of cells that were treated with the gyrase inhibitors.

Effect of thymine starvation on RK2 localization. In *E. coli* strains auxotrophic for thymine, thymine starvation results in the inhibition of DNA replication as well as a number of other direct and indirect effects, including DNA breakage and the induction of the SOS response (1). DG76, a *thyA46* strain, was subjected to thymine starvation to determine the effect of thymine-mediated replication inhibition on plasmid localization. DG76(pZZ15) cells were grown overnight in LB supplemented with thymine and then diluted into M63 with or without thymine. DG76(pZZ15) cells grown in thymine were somewhat longer, up to 6 μm long, and had more foci than MG1655(pZZ15) cells (Fig. 1C; see also Fig. 5A). After 6 h without thymine, however, many cells had become highly filamentous, reaching as much as 25 μm in length (Fig. 4A and 5A and B). These cells also contained up to 14 plasmid foci, with an average of 5.5 per cell compared to 3.2 for cells supplemented with thymine, again suggesting some destabilization of plasmid clusters, as seen with nalidixic acid and novobiocin treatment. Like MG1655(pZZ15) cells treated with nalidixic acid or novobiocin, DG76(pZZ15) cells grown without thymine displayed dispersed foci (56%) (Fig. 4A, panel i, and G, panel i) and mid-cell grouping of foci (33%) (Fig. 4A, panel ii, and G, panel ii). A further 11% of thymine-starved cells displayed the spaced grouping phenotype seen after novobiocin addition (Fig. 4A, panel iii, and G, panel iii). As with cells treated with either gyrase inhibitor, plasmid foci were largely confined to the region occupied by the nucleoids (Fig. 4D and G).

Redistribution of plasmid foci in thymine-starved cells after the reintroduction of thymine. To observe the effect of cell recovery after thymine starvation on plasmid localization, thymine was added back to cells after 6 h of starvation. One hour after the addition of thymine, foci had largely become dispersed throughout the cell (Fig. 4B and H). At this time point, only 5% and 2% of cells counted displayed mid-cell and spaced groupings of foci, respectively. Instead, the plasmid foci in most cells were more evenly distributed throughout the cells and within the nucleoid regions (Fig. 4E and H). At 2 h after the addition of thymine, foci appeared to be evenly distributed throughout the cell (Fig. 4C and I, panels i and ii). In addition, cell division had apparently resumed, as single cells were observed separating from the ends of the bacterial filaments and there was a high proportion of cells (48%) of unit length, with the remainder being filaments of various lengths.

Analysis of foci number and cell length at 3 h after the addition of thymine indicated the emergence of a large proportion of cells (Fig. 5C) that were of a length and foci count similar to cells that have not been deprived of thymine (Fig. 5A). Thus, the addition of thymine to thymine-starved cultures appears to result, in a large proportion of cells, in the redistribution of nucleoids and plasmid foci to their characteristically normal locations and the resumption of cell division.

Localization of the replisome with EYFP-RarA. It has been reported that novobiocin addition to *Caulobacter crescentus* results in a rapid disassembly of the replisome (33). In view of

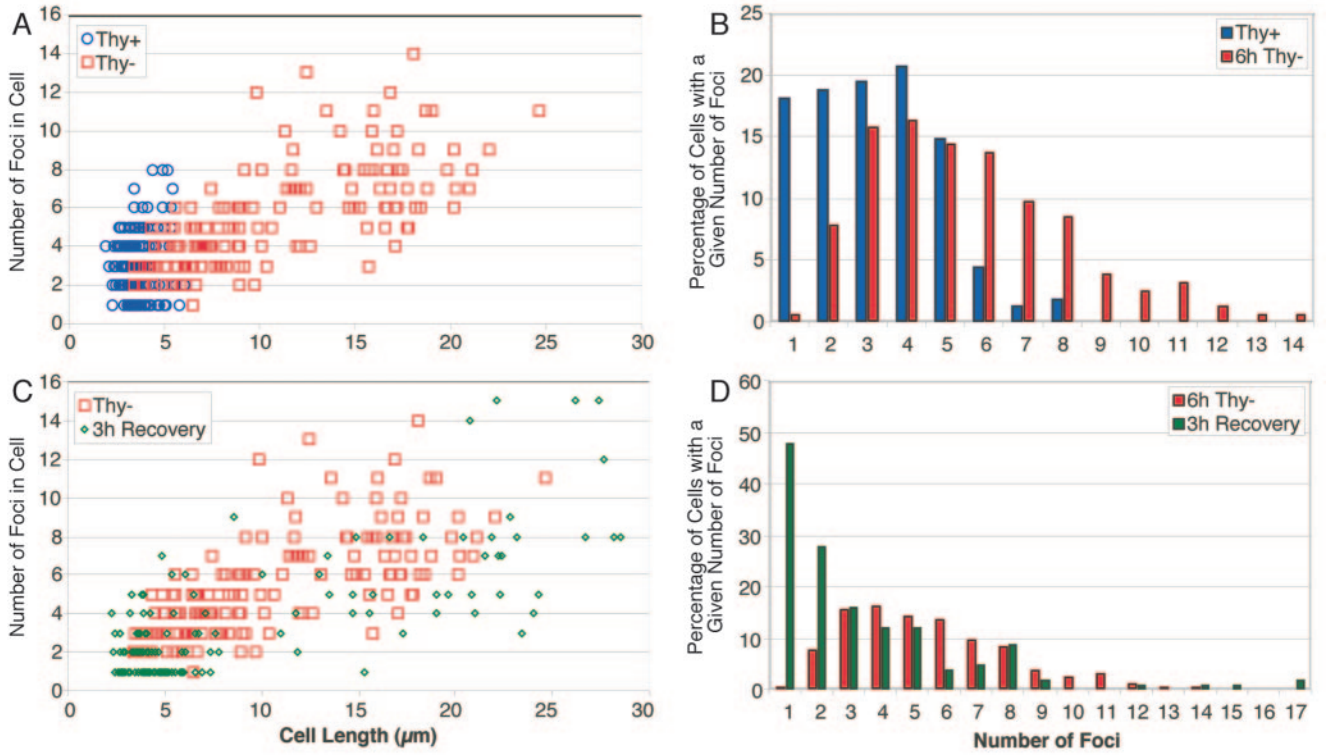

FIG. 5. Effect of thymine starvation of cells on foci number. (A and B) DG76(pZZ15) cells were supplemented with thymine (blue) or were grown without thymine for 6 h (red). (C and D) Cells grown without thymine for 6 h (red) and with thymine added after 6 h and then grown for an additional 3 h (green). Graphs show foci per cell versus cell length in micrometers (A and C) or the percentage of cells with a given number of foci (B and D). In panels A and C, each point represents one cell. A total of 154 thy$^+$, 152 thy$^-$, and 180 recovering thy$^-$ cells were counted. Of the thymine-starved cells that were stained with DAPI, 60% contained a single nucleoid. The remaining cells contained two nucleoids. The results are from a single experiment, but the thymine starvation was carried out a second time with overall similar results.

the possible role of the replication complex in plasmid cluster formation and localization, it was of interest to determine if novobiocin and/or nalidixic acid cause a similar disassembly of the replisome in *E. coli* MG1655. Evidence has been obtained for an association of the *E. coli* RarA protein with the replisome and can be used as an EYFP-RarA fusion protein to localize the replication complex by fluorescence microscopy (2, 38). MG1655 cells carrying pBS8, which expresses EYFP-RarA in response to L-arabinose, were treated with either nalidixic acid or novobiocin after the appearance of RarA foci (usually 1 to 2 h after the addition of L-arabinose to exponentially growing cells). Samples were removed over a 60-min period to determine the effect of addition of each of these two gyrase inhibitors on the EYFP-RarA foci. Novobiocin reduced the proportion of cells that exhibited one or more foci from 76% to less than 4% within 5 minutes after the addition of the inhibitor (Fig. 6A). Under the same growth conditions, novobiocin did not reduce the number of cells exhibiting foci consisting of GFP-LacI-tagged RK2 plasmid (Fig. 6B). Greater than 95% of the cells displayed RK2 foci before and after treatment with novobiocin over the 60-min period. In addition, the number of RK2 foci per cell did not increase substantially (Fig. 6C and E) despite the disassembly of the replisome, suggesting that a fully intact replication complex is not a critical factor in the clustering of individual RK2 molecules. A similar result was obtained after nalidixic acid addition, except that the RarA-tagged replication complexes reappeared in most cells after 30 to 60 min of treatment (Fig. 6A). Again, the addition of nalidixic acid did not result in a change in the proportion of cells exhibiting RK2 foci, or in a substantial change in the number of foci per cell, during the 60-min treatment despite the substantial loss of RarA foci during the initial 30 min after nalidixic acid addition (Fig. 6C and D). The reappearance of RarA foci after 30 to 60 min of nalidixic acid treatment may be due to repair synthesis of double-strand breaks, which are known to occur after addition of this gyrase inhibitor. As discussed previously, both nalidixic acid and novobiocin cause some RK2 cluster destabilization after 3 or 4 h of treatment, but neither shows significant destabilization within the first hour of treatment.

DISCUSSION

Both the chromosomal replication origin and plasmids exhibit specific localization and dynamic movement during bacterial cell growth and division (for reviews, see references 17, 24, and 56). After duplication, clusters of plasmids P1, F, R1, and RK2 have been shown to separate rapidly to specific locations at some time prior to cell division (16, 18, 31, 44, 49, 53). It has been estimated that plasmids P1 and F in *E. coli* separate five times faster than *oriC* (18). While the machinery for chromosomal origin movement and plasmid segregation

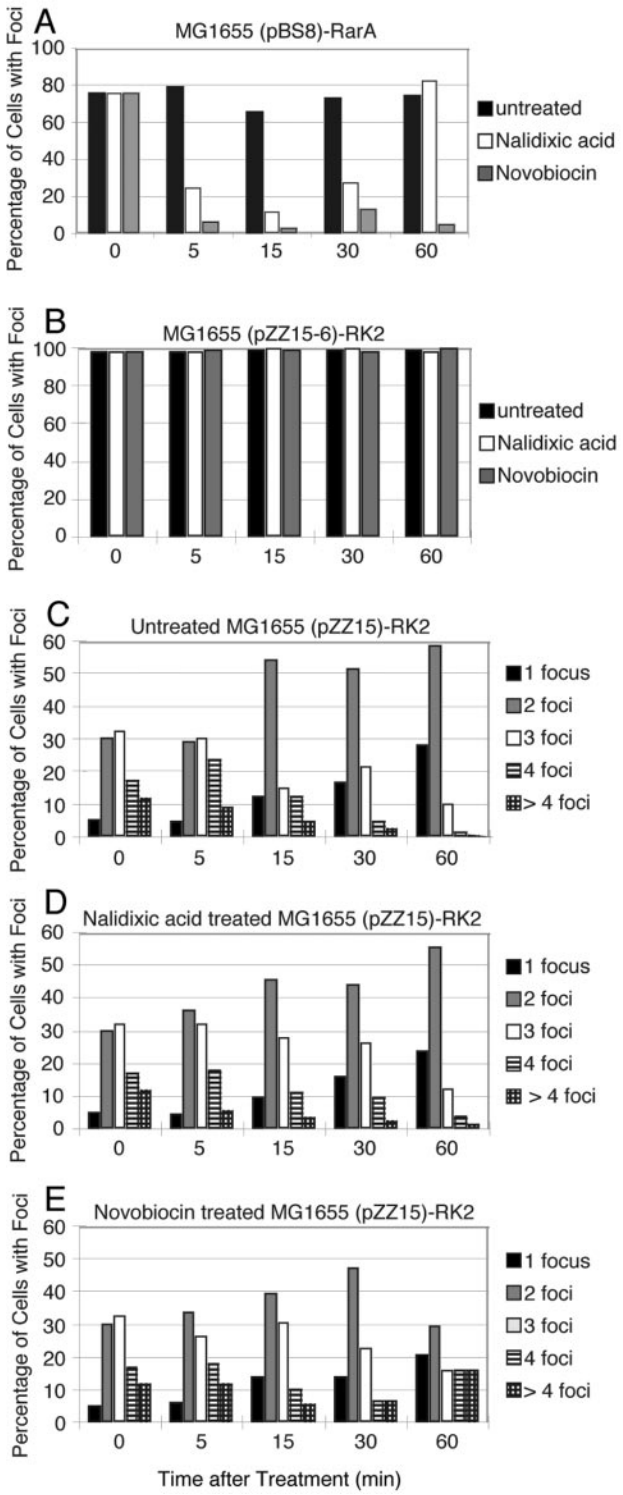

FIG. 6. RarA and RK2 foci in nalidixic acid- and novobiocin-treated cells. (A) Histogram of the percentage of cells with RarA foci at a given time after treatment in MG1655(pBS8) cells either untreated or treated with nalidixic acid or novobiocin. (B) Histogram of the percentage of cells with plasmid RK2 foci at a given time after treatment in MG1655(pZZ15) cells either untreated or treated with nalidixic acid or novobiocin. (C to E) Percentage of MG1655(pZZ15) cells with one, two, three, four, or greater than four foci at a given time in untreated cells (C), cells treated with nalidixic acid (D), or in cells treated with novobiocin (E).

may be the same or share common elements, it is clear that the separation of chromosomal origins and plasmid clusters are not coordinated with each other at a specific time in the cell cycle (15, 18, 25). Little is known about the role of plasmid- or host-encoded factors in clustering, localization, and movement of plasmids, but several recent studies have begun to provide some insight. Evidence has been obtained for a role of the MreB and the SetB proteins in chromosome segregation (7, 37). In the case of plasmid R1 the plasmid-encoded ParM protein, a member of the F-actin family, is likely to play a key role in segregation (32, 47). The partition systems of plasmids P1, F, and RK2 do not appear to encode actin-like proteins. The partition systems for these plasmids, however, do play a role in their specific localization (4, 6, 49). On the other hand, plasmid cluster formation occurs with or without a functional partition system (25, 64). While the par region of a plasmid does not appear to play a role in plasmid clustering, it is not unreasonable to expect that the plasmid replication origin with or without an associated plasmid-specified initiation protein is important in plasmid clustering.

It is likely that both plasmid-encoded and host factors contribute to plasmid clustering. There is accumulating evidence that both in *E. coli* and *B. subtilis* the DNA replication complexes are fixed in the region of the mid- and quarter-cell positions (36, 41, 42). It has also been demonstrated for the *E. coli* chromosome that in fast-growing cells, new replisomes are assembled and replication is initiated at the quarter positions, even before termination at the mid-cell replisome and before cell division (38, 40). For plasmids, however, it is not yet known if there is movement of the fully formed replication assembly from the mid- to the quarter-cell positions or if the assembly is reformed at the quarter-cell positions after disassembly at the mid-cell position. Nor is it known whether or not one or more components of the replication factory play a role in plasmid clustering, localization, and movement. Studies with plasmid pSC101 in *E. coli* supported a role for DNA replication proteins in segregation of this plasmid (46). In addition, it is conceivable that a plasmid cluster nucleates a replication complex that is dedicated specifically to the replication of that plasmid element. In *C. crescentus*, using a fusion of the δ′ component of DNA polymerase III to GFP to identify the position of the replication complex, additional foci were observed in cells that harbored a plasmid element (33). Similarly, it was recently reported that *B. subtilis* cells containing clusters of a multicopy plasmid also contained additional foci that were tagged with GFP fused to a subunit of DNA polymerase (62). The fact that the clusters of different replicons occupy different positions at the mid- or quarter-cell locations (25) may be the result of targeting of individual replication complexes with associated plasmid clusters to distinguishable locations within these regions of the cell.

The present study was directed at determining the effect of gyrase inhibition and/or the inhibition of DNA synthesis and, possibly, the disassembly of replication complexes on plasmid clustering and localization. It has been shown in vitro that the inhibition of gyrase activity prevents the initiation of plasmid RK2 replication (34). The fact that both of the gyrase inhibitors used in this study at best cause only partial declustering of plasmid pZZ15, which is present at five to eight copies per chromosome (9, 55, 59, 60), suggests that a normal level of

plasmid superhelicity is not a requirement for plasmid clustering. It is of course possible that there is sufficient residual superhelicity in plasmid pZZ15 after addition of the gyrase inhibitors to retain plasmid clustering. It is well established that DNA gyrase inhibition and thymine starvation of an *E. coli* thymine auxotroph inhibit DNA synthesis and indirectly inhibit cell division. After several hours the filaments that are produced by these treatments contain one or two extended nucleoids along the mid-line of the cell (Fig. 3 and 4). In the present study, using GFP-LacI to tag RK2 containing an array of *lacO* sequences, it was found that shutting down DNA synthesis interferes with the localization of plasmid foci at their characteristic mid- or quarter-cell position as the cell elongates prior to cell division. Under these conditions the foci are disordered but remain confined to the region of the nucleoid body, as revealed by DAPI staining. This disruption of the orderly spacing of both nucleoids and RK2 foci is not simply due to filamentation, since cephalexin treatment, which inhibits cell division but not replication, results in orderly spacing of nucleoids and foci clusters (Fig. 3). In addition, gyrase inhibition by the addition of nalidixic acid or novobiocin and thymine starvation result in a small increase in the number of foci per cell without a significant increase in the number of nucleoids, suggesting some declustering. On the basis of RK2 copy number, complete declustering of the plasmid would yield 5 to 8 foci in young cells and 10 to 16 foci in older cells prior to cell division. Interestingly, the readdition of thymine to thymine-starved cells results in a relocalization of plasmid foci over time and a reduction in the number of foci per cell, suggesting a reversal of the partial declustering and movement of the plasmid clusters to their normal fixed positions (Fig. 4).

Because of the direct and indirect effects of nalidixic acid, novobiocin, and thymine starvation on cell processes, the immediate cause of foci delocalization and partial declustering is not clear. Direct effects of thymine starvation, in addition to inhibition of DNA synthesis, are the formation of single- and double-strand breaks (10, 65), the induction of the SOS system (28, 29), and activation of DNA repair processes (see reference 1 for review). Indirect effects include inhibition of cell division (29) and filamentation (3). In the cases of novobiocin and nalidixic acid treatment, an immediate effect is the inhibition of gyrase and, therefore, a reduction of negative superhelical turns in plasmid and chromosomal DNA (12, 13, 58). In addition, as shown in Fig. 1A and reported by others (23, 57), there is a rapid inhibition of DNA synthesis by these two inhibitors and eventually an inhibition of cell division. Both nalidixic acid and novobiocin also inhibit *E. coli* topoisomerase IV (20, 26, 35). By virtue of its mode of action on *E. coli* gyrase, nalidixic acid also produces an irreversible lesion in DNA, which can lead to double-stranded breaks (22, 27). Unlike nalidixic acid, the coumarin novobiocin is a competitive inhibitor of the ATPase reaction of gyrase, and this inhibition does not result in the extensive formation of double-stranded breaks in DNA. It is of interest that despite the different modes of action and consequences with respect to the intactness of the DNA, similar effects on localization and clustering were obtained. This may suggest that inhibition of DNA synthesis in growing cells, a consequence of each of these treatments, is responsible for the changes observed in the localization of plasmid ZZ15. This difference in the extent of DNA breakage depending on the gyrase inhibitor used is consistent with the differential effect of these two gyrase inhibitors on the appearance of RarA foci (2, 38). Inactivation of the *rarA* gene in otherwise-wild-type cells does not result in a mutant phenotype; however, there is evidence that the RarA protein is associated with the replication complex in addition to being implicated in recombination events at the replication fork (2, 38). As shown in Fig. 6, the addition of either nalidixic acid or novobiocin causes a dramatic loss of RarA foci within 5 minutes of treatment. However, unlike novobiocin treatment, most of the nalidixic acid-treated cells began to redisplay RarA foci between 30 and 60 min after addition of the inhibitor. This reappearance of RarA foci in nalidixic acid-treated cells may be due to the production of double-stranded breaks and the attempts of the cell to repair these DNA breaks. At least for bacteriophage T4 there is a tight linkage between DNA replication and double-strand break repair in the life cycle of this bacteriophage in *E. coli* (14). The reappearance of RarA foci may thus also be related to attempts on the part of the cell to restart stalled replication forks, a process that may involve the induction of the SOS system and an accumulation of enzymes at the replication fork, including a replication complex.

The rapid disappearance of RarA foci after novobiocin or nalidixic acid treatment is interpreted as a partial or complete disassembly of the replisome as a direct or indirect result of inhibition of gyrase. In *C. crescentus* it was observed that novobiocin treatment caused the loss of replisomes tagged by a fusion protein consisting of GFP and the core δ′ subunit of DNA polymerase III (33), suggesting that treatment with this gyrase inhibitor results in more than just a loss of the RarA component of the replication factory in our experiments. Significantly, although there is disordering of the GFP-LacI-tagged RK2 foci, treatment with either gyrase inhibitor does not result in a significant increase in the overall number of foci or the distribution of foci per cell within 60 min (except for perhaps a somewhat higher number of cells with four or more foci after 60 min of treatment with novobiocin). Three to four hours of treatment is required to see an approximate doubling of the average number of foci, which is far less than a five- to eightfold increase that would be observed if the plasmid were completely declustered. These results suggest that while a fully intact replisome may play a roll in the targeting of the plasmid cluster to a specific location, it is not an essential factor in the clustering of plasmids. The disassembly of the replication factory, however, could result in an untethering of the plasmid cluster at its receptor site. Nevertheless, while the plasmid foci become disordered they remain confined to the region of the nucleoid body, even in substantially elongated filaments. This confinement may be due to a specific or nonspecific attachment of the plasmid cluster to the chromosomal matrix or a physical barrier to movement of detached plasmid clusters beyond the nucleoid body. The recent report of the reorganization of chromosomal DNA into ordered toroidal structures that are highly condensed and exhibit crystallographic features when *E. coli* cells enter the stationary state (11) may be relevant in this regard. It is possible that not unlike this effect of the stationary phase, a highly condensed state of chromosomal DNA occurs after gyrase inhibition and thymine starvation that may prevent the free movement of detached plasmid clusters and their confinement to the nucleoid body. Studies with plas-

mids P1 and R1 that lack functional partitioning genes and an *oriC* plasmid have shown the presence of plasmid foci outside of the nucleoid under normal growth conditions, indicating that plasmids are not in every case confined to the nucleoid body (6, 31, 49). It is of course possible that the partitioning genes of plasmid RK2 similarly are required for the normal positioning of this plasmid within the nucleoid body. The continued use of inhibitors that disrupt plasmid dynamics and the analysis of host and plasmid mutants that are defective in plasmid localization and movement should eventually provide a mechanistic insight into these complex processes.

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REFERENCES

1. **Ahmad, S. I., S. H. Kirk, and A. Eisenstark.** 1998. Thymine metabolism and thymineless death in prokaryotes and eukaryotes. Annu. Rev. Microbiol. **52:**591–625.
2. **Barre, F. X., B. Soballe, B. Michel, M. Aroyo, M. Robertson, and D. Sherratt.** 2001. Circles: the replication-recombination-chromosome segregation connection. Proc. Natl. Acad. Sci. USA **98:**8189–8195.
3. **Bazill, G. W.** 1967. Lethal unbalanced growth in bacteria. Nature **216:**346–349.
4. **Bignell, C. R., A. S. Haines, D. Khare, and C. M. Thomas.** 1999. Effect of growth rate and *incC* mutation on symmetric plasmid distribution by the IncP-1 partitioning apparatus. Mol. Microbiol. **34:**205–216.
5. **Ebersbach, G., and K. Gerdes.** 2004. Bacterial mitosis: partitioning protein ParA oscillates in spiral-shaped structures and positions plasmids at mid-cell. Mol. Microbiol. **52:**385–398.
6. **Erdmann, N., T. Petroff, and B. E. Funnell.** 1999. Intracellular localization of P1 ParB protein depends on ParA and *parS*. Proc. Natl. Acad. Sci. USA **96:**14905–14910.
7. **Espeli, O., P. Nurse, C. Levine, C. Lee, and K. J. Marians.** 2003. SetB: an integral membrane protein that affects chromosome segregation in *Escherichia coli*. Mol. Microbiol. **50:**495–509.
8. **Fangman, W. L.** 1969. Specificity and efficiency of thymidine incorporation in *Escherichia coli* lacking thymidine phosphorylase. J. Bacteriol. **99:**681–687.
9. **Figurski, D. H., R. Meyer, and D. R. Helinski.** 1979. Suppression of ColE1 replication properties by the IncP-1 plasmid RK2 in hybrid plasmids constructed *in vitro*. J. Mol. Biol. **133:**295–318.
10. **Freifelder, D.** 1969. Single-strand breaks in bacterial DNA associated with thymine starvation J. Mol. Biol. **45:**1–7.
11. **Frenkiel-Krispin, D., I. Ben-Avraham, J. Englander, E. Shimoni, S. G. Wolf, and A. Minsky.** 2004. Nucleoid restructuring in stationary-state bacteria. Mol. Microbiol. **51:**395–405.
12. **Gellert, M., K. Mizuuchi, M. H. O'Dea, T. Itoh, and J. I. Tomizawa.** 1977. Nalidixic acid resistance: a second genetic character involved in DNA gyrase activity. Proc. Natl. Acad. Sci. USA **74:**4772–4776.
13. **Gellert, M., M. H. O'Dea, T. Itoh, and J. Tomizawa.** 1976. Novobiocin and coumermycin inhibit DNA supercoiling catalyzed by DNA gyrase. Proc. Natl. Acad. Sci. USA **73:**4474–4478.
14. **George, J. W., B. A. Stohr, D. J. Tomso, and K. N. Kreuzer.** 2001. The tight linkage between DNA replication and double-strand break repair in bacteriophage T4. Proc. Natl. Acad. Sci. USA **98:**8290–8297.
15. **Gerdes, K., J. Moller-Jensen, G. Ebersbach, T. Kruse, and K. Nordstrom.** 2004. Bacterial mitotic machineries. Cell **116:**359–366.
16. **Gordon, G. S., D. Sitnikov, C. D. Webb, A. Teleman, A. Straight, R. Losick, A. W. Murray, and A. Wright.** 1997. Chromosome and low copy plasmid segregation in *E. coli*: visual evidence for distinct mechanisms. Cell **90:**1113–1121.
17. **Gordon, G. S., and A. Wright.** 2000. DNA segregation in bacteria. Annu. Rev. Microbiol. **54:**681–708.
18. **Gordon, S., J. Rech, D. Lane, and A. Wright.** 2004. Kinetics of plasmid segregation in *Escherichia coli*. Mol. Microbiol. **51:**461–469.
19. **Guzman, L. M., D. Belin, M. J. Carson, and J. Beckwith.** 1995. Tight regulation, modulation, and high-level expression by vectors containing the arabinose P_{BAD} promoter. J. Bacteriol. **177:**4121–4130.
20. **Hardy, C. D., and N. R. Cozzarelli.** 2003. Alteration of. *Escherichia coli* topoisomerase IV to novobiocin resistance. Antimicrob. Agents Chemother. **47:**941–947.
21. **Hashimoto-Gotoh, T., and M. Sekiguchi.** 1977. Mutations of temperature sensitivity in R plasmid pSC101. J. Bacteriol. **131:**405–412.
22. **Hiasa, H., D. O. Yousef, and K. J. Marians.** 1996. DNA strand cleavage is required for replication fork arrest by a frozen topoisomerase-quinolone-DNA ternary complex. J. Biol. Chem. **271:**26424–26429.
23. **Hill, W. E., and W. L. Fangman.** 1973. Single-strand breaks in deoxyribonucleic acid and viability loss during deoxyribonucleic acid synthesis inhibition in *Escherichia coli*. J. Bacteriol. **116:**1329–1335.
24. **Hiraga, S.** 2000. Dynamic localization of bacterial and plasmid chromosomes. Annu. Rev. Genet. **34:**21–59.
25. **Ho, T. Q., Z. Zhong, S. Aung, and J. Pogliano.** 2002. Compatible bacterial plasmids are targeted to independent cellular locations in *Escherichia coli*. EMBO J. **21:**1864–1872.
26. **Hoshino, K., A. Kitamura, I. Morrissey, K. Sato, J. Kato, and H. Ikeda.** 1994. Comparison of inhibition of *Escherichia coli* topoisomerase IV by quinolones with DNA gyrase inhibition. Antimicrob. Agents Chemother. **38:**2623–2627.
27. **Howard, M. T., S. H. Neece, S. W. Matson, and K. N. Kreuzer.** 1994. Disruption of a topoisomerase-DNA cleavage complex by a DNA helicase. Proc. Natl. Acad. Sci. USA **91:**12031–12035.
28. **Howe, W. E., and D. W. Mount.** 1975. Production of cells without deoxyribonucleic acid during thymidine starvation of *lexA*$^-$ cultures of *Escherichia coli* K-12. J. Bacteriol. **124:**1113–1121.
29. **Huisman, O., and R. D'Ari.** 1981. An inducible DNA replication-cell division coupling mechanism in *E. coli*. Nature **290:**797–799.
30. **Ishino, F., and M. Matsuhashi.** 1981. Peptidoglycan synthetic enzyme activities of highly purified penicillin-binding protein 3 in *Escherichia coli*: a septum-forming reaction sequence. Biochem. Biophys. Res. Commun. **101:**905–911.
31. **Jensen, R. B., and K. Gerdes.** 1999. Mechanism of DNA segregation in prokaryotes: ParM partitioning protein of plasmid R1 co-localizes with its replicon during the cell cycle. EMBO J. **18:**4076–4084.
32. **Jensen, R. B., and K. Gerdes.** 1997. Partitioning of plasmid R1. The ParM protein exhibits ATPase activity and interacts with the centromere-like ParR-*parC* complex. J. Mol. Biol. **269:**505–513.
33. **Jensen, R. B., S. C. Wang, and L. Shapiro.** 2001. A moving DNA replication factory in *Caulobacter crescentus*. EMBO J. **20:**4952–4963.
34. **Jiang, Y., J. Pogliano, D. R. Helinski, and I. Konieczny.** 2002. ParE toxin encoded by the broad-host-range plasmid RK2 is an inhibitor of *Escherichia coli* gyrase. Mol. Microbiol. **44:**971–979.
35. **Khodursky, A. B., E. L. Zechiedrich, and N. R. Cozzarelli.** 1995. Topoisomerase IV is a target of quinolones in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **92:**11801–11805.
36. **Koppes, L. J., C. L. Woldringh, and N. Nanninga.** 1999. *Escherichia coli* contains a DNA replication compartment in the cell center. Biochimie **81:**803–810.
37. **Kruse, T., J. Moller-Jensen, A. Lobner-Olesen, and K. Gerdes.** 2003. Dysfunctional MreB inhibits chromosome segregation in *Escherichia coli*. EMBO J. **22:**5283–5292.
38. **Lau, I. F., S. R. Filipe, B. Soballe, O. A. Okstad, F. X. Barre, and D. J. Sherratt.** 2003. Spatial and temporal organization of replicating *Escherichia coli* chromosomes. Mol. Microbiol. **49:**731–743.
39. **Lawley, T. D., and D. E. Taylor.** 2003. Characterization of the double-partitioning modules of R27: correlating plasmid stability with plasmid localization. J. Bacteriol. **185:**3060–3067.
40. **Lemon, K. P., and A. D. Grossman.** 2001. The extrusion-capture model for chromosome partitioning in bacteria. Genes Dev. **15:**2031–2041.
41. **Lemon, K. P., and A. D. Grossman.** 1998. Localization of bacterial DNA polymerase: evidence for a factory model of replication. Science **282:**1516–1519.
42. **Lemon, K. P., and A. D. Grossman.** 2000. Movement of replicating DNA through a stationary replisome. Mol. Cell **6:**1321–1330.
43. **Lewis, R. J., O. M. Singh, C. V. Smith, T. Skarzynski, A. Maxwell, A. J. Wonacott, and D. B. Wigley.** 1996. The nature of inhibition of DNA gyrase by the coumarins and the cyclothialidines revealed by X-ray crystallography. EMBO J. **15:**1412–1420.
44. **Li, Y., and S. Austin.** 2002. The P1 plasmid in action: time-lapse photomicroscopy reveals some unexpected aspects of plasmid partition. Plasmid **48:**174–178.
45. **Li, Y., and S. Austin.** 2002. The P1 plasmid is segregated to daughter cells by a "capture and ejection" mechanism coordinated with *Escherichia coli* cell division. Mol. Microbiol. **46:**63–74.
46. **Miller, C., and S. N. Cohen.** 1999. Separate roles of *Escherichia coli* replication proteins in synthesis and partitioning of pSC101 plasmid DNA. J. Bacteriol. **181:**7552–7557.
47. **Moller-Jensen, J., J. Borch, M. Dam, R. B. Jensen, P. Roepstorff, and K. Gerdes.** 2003. Bacterial mitosis: ParM of plasmid R1 moves plasmid DNA by an actin-like insertional polymerization mechanism. Mol. Cell **12:**1477–1487.
48. **Moller-Jensen, J., R. B. Jensen, J. Lowe, and K. Gerdes.** 2002. Prokaryotic DNA segregation by an actin-like filament. EMBO J. **21:**3119–3127.
49. **Niki, H., and S. Hiraga.** 1997. Subcellular distribution of actively partitioning F plasmid during the cell division cycle in *E. coli*. Cell **90:**951–957.
50. **Nordstrom, K., and K. Gerdes.** 2003. Clustering versus random segregation of plasmids lacking a partitioning function: a plasmid paradox? Plasmid **50:**95–101.

51. **Onogi, T., T. Miki, and S. Hiraga.** 2002. Behavior of sister copies of mini-F plasmid after synchronized plasmid replication in *Escherichia coli* cells. J. Bacteriol. **184:**3142–3145.
52. **Pardee, A. B., F. Jacob, and J. Monod.** 1959. The genetic control and cytoplasmic expression of "inducibility" in the synthesis of β-galactosidase by *E. coli*. J. Mol. Biol. **1:**165–178.
53. **Pogliano, J., T. Q. Ho, Z. Zhong, and D. R. Helinski.** 2001. Multicopy plasmids are clustered and localized in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **98:**4486–4491.
54. **Reich, Z., E. J. Wachtel, and A. Minsky.** 1994. Liquid-crystalline mesophases of plasmid DNA in bacteria. Science **264:**1460–1463.
55. **Rosche, T. M., A. Siddique, M. Larsen, and D. Figurski.** 2000. Incompatibility protein IncC and global regulator KorB interact in active partition of promiscuous plasmid RK2. J. Bacteriol. **182:**6014–6026.
56. **Sherratt, D. J.** 2003. Bacterial chromosome dynamics. Science **301:**780–785.
57. **Sugino, A., N. P. Higgins, P. O. Brown, C. L. Peebles, and N. R. Cozzarelli.** 1978. Energy coupling in DNA gyrase and the mechanism of action of novobiocin. Proc. Natl. Acad. Sci. USA **75:**4838–4842.
58. **Sugino, A., C. L. Peebles, K. N. Kreuzer, and N. R. Cozzarelli.** 1977. Mechanism of action of nalidixic acid: purification of *Escherichia coli nalA* gene product and its relationship to DNA gyrase and a novel nicking-closing enzyme. Proc. Natl. Acad. Sci. USA **74:**4767–4771.
59. **Thomas, C. M., M. A. Cross, A. A. K. Hussain, and C. A. Smith.** 1984. Analysis of copy number control elements in the region of the vegetative replication origin of the broad host range plasmid RK2. EMBO J. **3:**57–63.
60. **Thomas, C. M., R. Meyer, and D. R. Helinski.** 1980. Regions of broad-host-range plasmid RK2 which are essential for replication and maintenance. J. Bacteriol. **141:**213–222.
61. **van den Ent, F., J. Moller-Jensen, L. A. Amos, K. Gerdes, and J. Lowe.** 2002. F-actin-like filaments formed by plasmid segregation protein ParM. EMBO J. **21:**6935–6943.
62. **Wang, J. D., M. E. Rokop, M. M. Barker, N. R. Hanson, and A. D. Grossman.** 2004. Multicopy plasmids affect replisome positioning in *Bacillus subtilis*. J. Bacteriol. **186:**7084–7090.
63. **Weitao, T., S. Dasgupta, and K. Nordstrom.** 2000. Plasmid R1 is present as clusters in the cells of *Escherichia coli*. Plasmid **43:**200–204.
64. **Weitao, T., S. Dasgupta, and K. Nordstrom.** 2000. Role of the *mukB* gene in chromosome and plasmid partition in *Escherichia coli*. Mol. Microbiol. **38:** 392–400.
65. **Yoshinaga, K.** 1973. Double-strand scission of DNA involved in thymineless death of *Escherichia coli* 15 TAU. Biochim. Biophys. Acta **294:**204–213.