Emergence of antibiotic resistance from multinucleated bacterial filaments

Julia Bos\textsuperscript{a}, Qiucen Zhang\textsuperscript{b}, Saurabh Vyawahare\textsuperscript{a}, Elizabeth Rogers\textsuperscript{c}, Susan M. Rosenberg\textsuperscript{c}, and Robert H. Austin\textsuperscript{a,1}

\textsuperscript{a}Department of Physics, Princeton University, Princeton, NJ 08544-0708; \textsuperscript{b}Department of Physics, University of Illinois at Urbana-Champaign, Urbana, IL 61801-3080; and \textsuperscript{c}Departments of Molecular and Human Genetics, Biochemistry and Molecular Biology, and Molecular Virology and Microbiology, and the Dan L. Duncan Cancer Center, Baylor College of Medicine, Houston, TX 77030

Contributed by Robert H. Austin, November 3, 2014 (sent for review August 26, 2014)

Bacteria can rapidly evolve resistance to antibiotics via the SOS response, a state of high-activity DNA repair and mutagenesis. We explore here the first steps of this evolution in the bacterium \textit{Escherichia coli}. Induction of the SOS response by the genotoxic antibiotic ciprofloxacin changes the \textit{E. coli} rod shape into multichromosome-containing filaments. We show that at subminimal inhibitory concentrations of ciprofloxacin the bacterial filament divides asymmetrically repeatedly at the tip. Chromosome-containing buds are made that, if resistant, propagate nonfilamenting progeny with enhanced resistance to ciprofloxacin as the parent filament dies. We propose that the multinucleated filament creates an environmental niche where evolution can proceed via generation of improved mutant chromosomes due to the mutagenic SOS response and possible recombination of the new alleles between chromosomes. Our data provide a better understanding of the processes underlying the origin of resistance at the single-cell level and suggest an analogous role to the eukaryotic aneuploidy condition in cancer.

antibiotic resistance | SOS response | filamentation | mutation | evolution

Bacteria can evolve remarkably quickly to be resistant to antibiotics under certain conditions, as we showed in an earlier paper (1) using microfabrication techniques to create a structured environment of bacteria in a gradient of the genotoxic antibiotic ciprofloxacin (cipro), which perturbs chromosome replication (2). The need for the structured environment in which steep gradients facilitate the rapid evolution of resistance by multiple mutations confirmed a prediction from recent modeling (3). Our earlier work on the rapid emergence of cipro resistance in a microfabricated complex ecology did not delineate the stages in the emergence of resistance but only presented the ultimate, resistant cells (1). However, high-resolution imaging of the cells within the device before the full emergence of resistance showed that the cells went through a transient filamentation phase. Fig. 1 shows images from our microecology that \textit{Escherichia coli} bacteria have changed their shape into long filamentous structures shortly after exposure to the cipro antibiotic. The filamentation response is not permanent, because after 4 h some cells have reverted to a normal phenotype (Fig. 1B) and have become resistant to high levels of the antibiotic by de novo mutation in genes encoding gyrase A topoisomerase and efflux pumps (1). We wished to explore the role filamentation played in the initial stages of antibiotic resistance.

Many microorganisms adopt a filamentous shape caused by cell-division arrest yet continue cell-volume growth in response to a variety of stressful environments, including nutrient deficiency (4), extensive DNA damage through the SOS response pathway (5, 6), host innate immune responses (7), desiccation (8), high pressure (9), and antibiotic treatment (10–12). Although filamentation has long been considered an overstressed and/or sick phenotype or solely for completion of DNA replication before division (13), it has only recently been described as a more general survival strategy (7, 8, 14). For example, if the stress is relieved quickly enough, division of the filamentous cell resumes synchronously and rapidly at regular intervals along the entire length of the filament, resulting in multiple viable normal-sized offspring cells (8). However, the mechanisms underlying the evolution of filamentous bacteria when the selective pressure is maintained have been poorly described, in part because most studies have used stress conditions that were evolutionarily unfavorable and instead led to irreversible bacterial growth arrest and/or rapid death.

Bacterial cell-division arrest resulting from inhibition of DNA replication after cipro exposure (2) is caused by the induction of a division inhibitor (e.g., SulA in \textit{E. coli}) (13, 15). sulA gene induction is part of a set of over 30 induced genes involved in DNA repair pathways and error-prone mechanisms, collectively known as the SOS response (5, 16, 17), a sequential transcriptional process that is tightly controlled by two master regulators, LexA and RecA proteins (18). When the replication forks are stalled, LexA autolytic cleavage facilitated by RecA protein initiates the SOS stress pathway until the chromosome damage has been repaired and reconnection of the chromosome resumes. However, when the SOS response is fully engaged, the induction of low-fidelity replication polymerases known as error-prone polymerases increases the rate of mutation \( \mu \) during DNA replication from the normally low value of \( 10^{-9} \) mutations per base pair generation up to a high rate of \( 10^{-5} \) mutations per base pair generation (19), so that a single bacterial chromosome such as the 4.6-Mb \textit{E. coli} chromosome can be expected to have multiple mutations with each round of replication. Current research aims at targeting the error-prone polymerase pathway to slow down the evolution of antibiotic resistance and thus improve the long-term viability of some antibiotic drugs (20).

Here we address how filamentation is probably the first step in the evolution of resistance to mutagenic antibiotic exposure, internally generating mutant chromosomes via the SOS response until a solution is obtained: a mutant chromosome with resistance to the stress. We examine how individual bacteria exit from the filamentous state caused by subminimal inhibitory concentrations of the genotoxic antibiotic ciprofloxacin. We show that when cipro is maintained at low levels, \textit{E. coli} filaments

Significance

Understanding how bacteria rapidly evolve under antibiotic selective pressure is crucial to controlling the development of resistant organisms. We show that initial resistance emerges from successful segregation of mutant chromosomes at the tips of filaments followed by budding of resistant progeny. We propose that the first stages of emergence of resistance occur via the generation of multiple chromosomes within the filament and are achieved by mutation and possibly recombination between the chromosomes.


The authors declare no conflict of interest.

1To whom correspondence should be addressed. Email: austin@princeton.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1420702111/-/DCSupplemental.
undergo asymmetric divisions at the filament tips, giving rise to budded resistant offspring cells. The generation of multiple mutant chromosomes that are successfully segregated toward the filaments tips favors the birth of antibiotic-resistant cells. We discuss how this filamentation step is a precursor to the final highly resistant bacteria in a complex ecology.

Results

We focused on the role that the SOS mutational network, triggered by the presence of stalled replication forks, plays in the filamentation process and bacterial evolution of drug resistance. The transient filamentation events were studied on agar plates containing cipro at varying levels. The primary experiment involves spreading a low-density population of less than 10^5 per mm^2 of E. coli bacteria in the presence of 0.125 of the minimal inhibitory antibiotic concentration (0.125×MIC) of cipro. Time-lapse imaging revealed that 99.5% of the bacteria formed long filaments (up to 200 μm) and expanded their length exponentially (Fig. 2A; see Movies S1 and S2 for videos of the growth of filaments). The remaining 0.5% of the bacteria that did not elongate or divide in the presence of the antibiotic may be persist cells (21) or dead cells. A lexA3 mutant strain that cannot activate the SOS response and a recB mutant strain, which reduces SOS response induction, general recombination, and DNA repair, were not able to filament or grow at these low cipro concentrations, verifying that both filamentation and growth rely on the induction of the LexA-dependent SOS response and possibly on recombination and repair pathways (SI Appendix, Fig. S1A shows the growth of filaments over a 3-h time period and the failure of the lexA3 and recB mutant strains to grow under the same conditions). We observed that the growth of a filament is transiently interrupted by asymmetric division events (Fig. 2B and Movies S1 and S2) that occur repeatedly and predominantly at the tips of the filament (Fig. 2C) and give rise to short offspring cells that often resumed normal division (Fig. 2B and Movies S1 and S2). This budding process was inherently distinct from the synchronous, symmetrical division recovery profile of the filament obtained shortly after drug removal (SI Appendix, Fig. S1).

Interestingly, some degree of heterogeneity was observed in the bud fate at this low concentration (0.125×MIC) (Fig. 3A): 35% of budded cells divide symmetrically without further filamentation, indicating that they are now resistant to 0.125×MIC cipro; 35% cease division and growth; 10% of the buds show abnormal asymmetrical division (minicell formation) with little propagation; and 20% resume filamentation, indicating that they do not show evidence of cipro resistance. Buds that fail to divide and propagate may either contain a damaged chromosome or may not contain a chromosome at all [we found that 11% of the buds do not contain a chromosome, by using 4′,6-diamidino-2-phenylindole (DAPI) staining]. Conversely, the fate of the filaments after prolonged growth on low cipro showed less heterogeneity than that of the buds, since the bulk of parental filaments (70%) undergo death as evidenced by their inability to exclude the propidium iodide (PI) dye, reflecting loss of possibly both membrane integrity and efflux mechanisms (Fig. 3B). These findings suggest that filamentation at low cipro is a complex phenomenon that gives rise to short progeny and eliminates the unfit filamentous parent cell.

We were most interested by the buds that divide symmetrically without further filamentation and examined whether these buds show a lower level of SOS response. We used a sulA-gfp transcriptional reporter in a nongenic chromosomal location in cells with an intact native sulA gene (22) to quantitatively measure induction of the SOS response within the bacterial filament. We observed a decrease in the intensity of the gfp expression within buds, indicating that buds had decreased expression of the SOS regulon, as would be expected from the observed increase in cipro resistance in the buds and subsequent suppression of the SOS response [Fig. 3 shows that the buds have on average about 25% less expression of Psula-gfp than the filaments, whereas the intensity of constitutively expressed RFP protein (lac-mrfp1) shows identical levels both in the buds and in the filaments, indicating that protein expression levels are similar in the buds and within the filaments].

Because most buds do contain a chromosome, although cipro directly affects DNA replication by targeting DNA gyrase (23) and thus perturbs chromosome dynamics (2), we wished to examine both the content and the spatial arrangement of the chromosomes in filamentous cells to understand how low doses of cipro allow the generation of short viable nucleated offspring. We used standard DAPI staining and live imaging of a specific chromosomal locus, R2 (17), as a reporter of an individual chromosomal copy (SI Appendix, Fig. S2) to enhance chromosome counting. R2 loci were visualized by the binding of fluorescent TetR-CFP fusions to the tetO operator array located 21 kb upstream of the lacZ gene (24) (SI Appendix, Fig. S2). Individual chromosomes localize as discrete CFP foci. We found...
that the filaments contain multiple chromosomes (Fig. 4 and SI Appendix, Fig. S2) that are partially or completely segregated, with a linear relation between filament length and chromosome number (Fig. 4A), indicating that as the filament grows it replicates multiple chromosomes. The presence of multinucleated filaments occurred only at a low level of cipro (0.125× MIC) (SI Appendix, Fig. S2C). At levels of cipro above 0.125× MIC, the chromosome morphology was severely impaired (SI Appendix, Fig. S2C) and events of budding at the filament tips were rare or nonexistent. At low levels of cipro, we found that some chromosomes localize near the tips (three-quarter positions of cell length) (Fig. 4B), coinciding with the position of the division sites at the tips (Fig. 1C). Time-lapse experiments showed heterogeneous dynamics of the chromosomes within the filaments where some chromosomes move tipward as the filaments grow whereas other remain essentially stationary in the midcell regions (Fig. 4C and D; SI Appendix, Fig. S3; and Movie S3). In addition, two individual chromosomes were occasionally observed between overlapping in a single cluster and then separating into two distinct foci (Fig. 4C and D and SI Appendix, Fig. S3). We also noticed that the chromosomes within the same filament undergo replication or separation of foci in an asynchronous way (Fig. 4C and D). All together, the chromosome dynamics in cipro-stressed filaments are complex and do not indicate simple replication, but nevertheless the filamentous cell succeeds in coordinating chromosome placement and asymmetric division at the tips, leading to the birth of resistant daughter cells.

We verified that the offspring evolved resistance in that normal-sized and dividing bacterial buds arose at the tips of the filaments under continued low cipro treatment, and SOS expression levels, as determined by the sulA-gfp reporter, were low. Wild-type bacteria were cultured in low cipro and filtered using a sterile 5-μm syringe filter to isolate normal-sized progeny from filamentous parent cells (SI Appendix, Fig. S4). About 70 ± 11% of the isolated progeny survived when reexposed to low cipro (0.125× MIC). The frequency of progeny resistant to high levels of cipro (1× MIC) was determined and defined as the number of cipro-resistant colonies that arose after 24 h on solid media containing 1× MIC cipro per viable cell. Progeny showed a 250-fold increase in resistance frequency [2.8 (±0.3) × 10⁻⁹] at 1× MIC cipro compared with that of the parent cells [7.2 (±3) × 10⁻¹⁰] that had not been preexposed to low cipro. This result indicates that a subpopulation of budded cells has indeed evolved resistance during the 24-h exposure to low cipro. We assumed that the frequency of persisters was kept low in the progeny population, even after filtration, because progeny either formed filaments or resumed normal division when exposed over again to low cipro. Instead, persisters would have resumed division and growth only once the antibiotic is removed (21).

Resistant clones were isolated by selection using subinhibitory (0.5× MIC) or inhibitory (1× MIC) doses of cipro, and a subset of genomic sequences of known resistant genes was analyzed by using targeted genome sequencing (Table 1 and SI Appendix, Table S2). The results showed that the level of resistance was a function of the cipro concentration at selection. Resistance at the bottom of the selection window was associated with over-expression of AcrAB efflux pumps, in that mutations in the sulA-gfp expression within filaments (Fig. 4B) and constitutively expressed rfp (Bottom) in the same cells after 4 h of growth in low cipro. (Scale bar, 2 μm.) (C) Microscopic images of filaments and buds expressing sulA-gfp (Top) and constitutively expressed rfp (Bottom) in the same cells after 4 h of growth in low cipro. (Scale bar, 2 μm.) (D) Histogram of average sulA-gfp expression within filaments (n = 98 cells). The red line represents a GFP mean intensity of 393 ± 122 of the population. (E) Histogram of average sulA-gfp expression within buds (n = 110 cells). The red line represents a GFP mean intensity of 393 ± 107 of the population.
mutation in gyrA (GyrA S83A), which is located in the quinolone resistance-determining region, a hot spot region for mutations (25). Mutations in gyrA or marR are likely to be sufficient to promote intermediate-level resistance of the tested clones (Fig. 5). The rest of the cipro-resistant clones presumably carry a resistance-conferring mutation(s) at a place not sequenced.

The MIC of resistant progeny was 2.5× increased (10 clones analyzed) but remained five times lower than that of the high-level resistant strain QZ030, which evolved four mutations both in gyrA and the efflux pumps when cultured in a complex environment (1). Although the budded progeny show enhanced resistance to cipro, they do not show the full resistance observed in the cells that grow in the microfabricated devices, indicating that the budded progeny are part-way along the path to a fully enhanced resistance (Fig. 5 compares the resistance of filtered bud progeny to cipro with that of the strain of bacteria QZ030 retrieved from our previous experiment, where evolution to resistance to cipro emerged in a complex gradient in a meta-population, as opposed to strains extracted from this experiment, where evolution occurs in a fixed concentration of cipro). In all, the data reveal that a low level of adaptive evolution to low cipro can occur in a few hours within a single filament.

**Discussion**

Our data show that there are five fundamental features that characterize the growth of the bacterial filaments during exposure to subminimal inhibitory concentrations of cipro (Fig. 6): (i) Exposure to 0.125× MIC cipro initiates the SOS response (Fig. 3C and Fig. S1); (ii) the filament contour length L(t) increases exponentially with time (Fig. 2A); (iii) the filaments contain multiple copies of the bacterial chromosome (Fig. 4); (iv) the filamenting process is transiently interrupted by a budding process that generates resistant progeny at the tips (Fig. 1 and Movies S1 and S2); and (v) most filaments die, but not some of the budded progeny (Fig. 3B).

Such an evolutionary path to antibiotic resistance encompasses two remarkable events that occur during the filamentation process: the presumably local decision to transiently exit from SOS and bud a resistant daughter cell from the end of the filament, and the generation of multiple chromosomes that evolve resistance via mutagenesis and possibly recombination within a single filament.

The budding events are presumably triggered by the local cessation of the SOS response in the vicinity of an evolved resistant chromosome with the mutations found in Table 1, because the SOS response is responsible for the filamentation phenotype caused by a lack of septation. It could be either that better-adapted chromosomes are generated near the tips or that better-adapted chromosomes generated internally migrate toward the tips, where they are then budded off by formation of a single septum. We can guess whether the cessation of the SOS response is a global or local event in the cell by estimating how far the SOS division-inhibitor SulA can diffuse during the replication time of a chromosome, about 30 min in our case. SulA has a molecular mass of about 18 kDa and measurements of the diffusion coefficient of similar-size GFP proteins in E. coli have yielded an average value for the diffusion constant of about 7 μm²/s (26), and hence we would expect that in 30 min the SulA protein should diffuse about 150 μm, easily the length of a filament. However, the budding process is local and not evenly distributed along the length of the filament, indicating that either SulA does not diffuse freely in the cytoplasm or other players such as the Min division control proteins are determining the local decision to bud. Identifying the molecular actors that govern the decision of local budding will be of valuable interest to answer the question of why asymmetric division is important to generating resistant progeny.

This view differs from current paradigms for cell-cycle checkpoint control in bacteria and other organisms. Cell filamentation during SOS is thought to reflect the need to complete DNA replication before cell division to protect chromosomes after DNA damage (13). Similarly, filamentation allows repair of DNA damage by recombination between chromosomes by keeping more than one chromosome per cell until repair is completed (27). Similar DNA-protection roles underlie eukaryotic cell-cycle checkpoint control (28). We suggest that in addition to DNA preservation, cell filamentation may accelerate evolution by adding recombination, which is possible only with more than one chromosome per cell, to the increased mutagenesis documented during SOS. Recombination between chromosomes can potentially separate beneficial mutations from linked deleterious mutations or unpaired DNA damage. The dramatic acceleration of evolution by recombination accompanying mutagenesis, relative to mutagenesis without recombination, is supported by modeling (29, 30).

Coupling of mutagenesis with recombination is observed throughout life, including being predicted in cancer genomes (31, 32), and observed in the immune system (33), yeast (34, 35) and bacteria (17, 36–38). Because increased mutagenesis creates both deleterious and beneficial mutations, recombination that unlinks these can be beneficial.

**Table 1.** Targeted-sequencing results showing genes mutated in resistant progeny selected at 0.5× MIC and 1× MIC cipro

<table>
<thead>
<tr>
<th>Gene</th>
<th>Cipro at selection, ng/mL</th>
<th>No. of mutants (base substitution)</th>
<th>No. of mutants (base deletion)</th>
</tr>
</thead>
<tbody>
<tr>
<td>gyra</td>
<td>20</td>
<td>0/16</td>
<td>0/16</td>
</tr>
<tr>
<td>gyra</td>
<td>40</td>
<td>7/8</td>
<td>0/8</td>
</tr>
<tr>
<td>marR</td>
<td>20</td>
<td>0/8</td>
<td>3/8 (marR Δ 1, Δ 11)</td>
</tr>
<tr>
<td>marR</td>
<td>40</td>
<td>0/8</td>
<td>0/8</td>
</tr>
<tr>
<td>acrR</td>
<td>20</td>
<td>0/8</td>
<td>0/8</td>
</tr>
<tr>
<td>acrR</td>
<td>40</td>
<td>0/8</td>
<td>0/8</td>
</tr>
<tr>
<td>rbsA</td>
<td>20</td>
<td>0/8</td>
<td>0/8</td>
</tr>
<tr>
<td>rbsA</td>
<td>40</td>
<td>0/8</td>
<td>0/8</td>
</tr>
<tr>
<td>sulA</td>
<td>20</td>
<td>0/4</td>
<td>0/4</td>
</tr>
</tbody>
</table>

The number of mutants over the number of sequenced clones (e.g., 4, 8, or 16) is indicated.

![Fig. 5.](image) Resistance of the wild-type strain, a QZ030 hyperresistant isolate (1), and three different progeny clones to cipro as measured by growth curves. The cipro concentration at which growth was monitored is indicated.
Further, the chromosomes display a distinct dynamics pattern in the same filament. Events of merging/splitting between two chromosomes were frequently observed during the experiment (Fig. 4C; SI Appendix, Fig. S3; and Movie S2), possibly reflecting the intimate interplay between DNA replication and genetic information exchange during cipro exposure. In addition, replication of sister loci did not occur simultaneously, as shown in Fig. 4C, where one chromosome out of six was captured while replicating, and suggests potential differential DNA damage load between the chromosomes (39). Although we found these observations captivating, it remains unclear to us why there is such heterogeneity in the chromosome dynamics. One can speculate that the nonstationary chromosomes are the chromosomes with increased fitness (probably containing mutations conferring cipro resistance) that would not be eliminated but chosen for replication. Instead, stationary and/or nonreplicating chromosomes that are likely to contain neutral or deleterious mutations would eventually be eliminated in the dying filament or would recombine to separate beneficial mutations from linked deleterious damage and hence be chosen for survival (Fig. 3B). Therefore, the multiple chromosomes that are generated in the filament could enhance the chances of producing resistant chromosomes as well as allow repair. The ultimate decision to release the progeny would be done by pinching off the successful mutant chromosome that is resistant to the stress by local loss of the SOS response. Further in-depth mechanistic studies of the interplay between processes of stress-induced mutagenesis, recombination, chromosome partitioning, and local budding are needed to deepen our understanding of how antibiotic resistance stems from a single multicellular bacterium. The chromosome dynamics in an elongating filament might possibly resemble a form of Turing machine (40), where the “computation” of fitness is like the writing of mutations on a chromosome and erasure of marks would correspond to the elimination of chromosomes that were less fit and to recombination between chromosomes.”

Materials and Methods

**Imaging.** Long time-lapse movies (images recorded every 2 min for 10 h) of low-density bacteria [JEK1036 (gfp::) growing on LB agar pads containing 5 ng/mL cipro and isopropyl β-D-1-thiogalactopyranoside (IPTG) were made using a Nikon 90× microscope equipped with a Nikon Plan Apo 60× objective and a Rolera-XR cooled CCD camera and processed with Micro-Manager (NIH, www.micro-manager.org) and ImageJ (NIH) software. All other images were collected on a Nikon Eclipse 90 microscope equipped with a Nikon Plan Apo 100× objective and an Andor Neo camera and processed with Nikon NIS-Elements software. In all experiments, cells were transferred from liquid culture to 1.4% agarose-padded slides containing LB medium (or 0.5× LB for strain AB1157), with low ciprofloxacin and IPTG when required. A coverslip was placed on the pad and then sealed with vasalp (1 vol vaseline/1 vol lanolin/1 vol paraffin). Filament death was visualized by fluorescence microscopy using propidium iodide, a membrane-impermeable nucleic acid stain commonly used as a cell-death marker. Nucleoids in the filaments were visualized by adding DAPI (live-cell DNA staining) directly to the agarose pads. R2 loci were visualized in E. coli AB1157 cells plated on 1.4% agarose-padded slides containing 0.5× diluted LB medium (to reduce fluorescence background). Images were recorded every 2 min right after the cells were placed on the agarose pad in the no-cipro conditions, or every 5 min after the cells were allowed 2 h of growth on the agarose pad in the low-cipro conditions. See SI Appendix, Materials and Methods for details of experimental protocols.

**Bacterial Strains and Growth.** E. coli strains were cultured at 37 °C in liquid LB medium and on an LB agar (1.7%) plate (solid), unless noted. In all experiments, cipro antibiotic was added at a subminimal inhibitory concentration of 5 ng/mL (0.125× MIC) referred to as “low cipro,” unless indicated (see SI Appendix, Materials and Methods for details of strain growth). Strains and primers used in this study are listed in SI Appendix, Tables S1 and S2, respectively.

**Minimum Inhibitory Concentration Determination.** The MIC defined as the lowest concentration of ciprofloxacin that prevents any growth of the bacteria is known to be 40 ng/mL. For each strain, a volume of 3 mL of overnight culture was used to inoculate in triplicate, 150 μL of LB medium containing increasing concentrations of ciprofloxacin (0, 5, 10, 15, 20, 25, 30, and 40 ng/mL) using 96-well flat-bottom plates. Growth was monitored by reading the absorbance at 630 nm every 20 min for 10 h of incubation at 37 °C with shaking in a ChromMate 4300 Microplate Reader (Awareness Technology). MICs for ciprofloxacin-resistant offspring clones (10 clones analyzed) were determined using the same conditions described above; in addition, wells contained LB plus ciprofloxacin at 50, 100, 200, and 500 ng/mL.

**Determination of Cipro-Resistant Mutant Frequency.** The frequency of the cipro-resistant (ciproR) mutant was determined and defined as the number of ciproR colonies that arose after 24 h of growth on solid media containing 1× MIC cipro, per viable cell. Three independent cultures of wild-type strains were subcultured at 1:100 dilution from an overnight starter culture and grown to midexponential phase. A volume of 0.1 μL of cell culture was plated on LB agar containing low cipro antibiotic and incubated at 37 °C for 24 h. The bacterial lawn was resuspended in 5 mL LB using a sterile scraper. The resulting cell culture contained a mixed population of filaments and short bud offspring. When needed, the cell culture was recovered by filtration through a 5-μm sterile syringe filter to harvest the progeny and remove the filamentous cells. All cultures (mixed-population or filtered progeny) were serially diluted, and 0.1-μL samples of appropriate dilutions were plated onto nonselective plates to determine total viable cell counts, as well as on plates containing cipro 1× MIC (40 ng/mL) to score high-level ciproR mutants. CiproR frequencies were calculated by dividing the number of high-level cipro-resistant mutants by the total number of viable bacteria. A total of three experiments was conducted independently. Means of mutant frequency are given in the text and the “±” values represent SD.

**Sequencing.** Cipro-resistant colonies were selected on LB plates containing either cipro 0.5× MIC (20 ng/mL) or 1× MIC (40 ng/mL), after 24 h incubation at 37 °C. See SI Appendix, Materials and Methods for details of the experimental protocol.

**ACKNOWLEDGMENTS.** We thank Thomas Silhavy for helpful comments on the manuscript and Douglas Austin for helping in writing the custom MATLAB code. This project was supported by National Cancer Institute Grant U54CA143803 (to R.H.A.) and National Institutes of Health Grant R01-GM53158 (to S.M.R.).


33. Donley N, Thayer MJ (2013) DNA replication timing, genome stability and cancer: Late and/or delayed DNA replication timing is associated with increased genomic instability. Semin Cancer Biol 23(2):80–89.


Movie S1. *Escherichia coli* (wild-type) bacteria growing in the presence of low cipro for 9 h (544 min). Microscopy images were taken using the GFP channel every 2 min. The movie’s frame rate is 15 images per s. (Scale bar, 10 μm.)
Movie S2. *E. coli* (wild-type) bacteria growing in the presence of low cipro for 9 h (544 min). Microscopy images (bright-field) were taken every 2 min. The movie's frame rate is 15 images per s. (Scale bar, 10 μm.)
Movie S3. Time course of chromosome (R2-CFP loci) localization in filamentous *E. coli* (wild-type) cells growing in the presence of low cipro. Images were taken every 5 min. The movie's frame rate is three images per s. (Scale bar, 2 μm.)
Other Supporting Information Files

SI Appendix (PDF)
Supplementary Material includes supplementary Figure and Movie captions, 2 tables, supplementary Methods and 4 supplementary Figures.
Supplementary Figure Captions:

Figure S1: Filamentation in low cipro requires the LexA-dependent SOS response activation.
A. Microscopy images of wild type (gfp+) and lexA3 (gfp+) mutant bacteria growing on LB agar containing low cipro. The time (min.) at which the images were taken is indicated. B. Microscopy images of division recovery along the filaments after 2.5h of cipro removal. Brightfield (top) and GFP (bottom) images are shown. White arrowheads indicate the multiple septation sites formed along the filament. In all images, scale bar is 2 microns.

Figure S2: Visualization of multiple chromosomes within single filaments
A. Schematic representation of the location of the fluorescent locus (R2) along the chromosome of strain AB1157 (1, 2). The blue triangle indicates the insertion position of R2 (tetO) operator arrays (20kb upstream the lacZ gene) used for chromosome localization. Replication origin (oriC) is shown, as well as the bidirectionality of the replication forks movement (grey arrows). Expression and binding of TetR-CFP onto the tetO array give rise to discrete fluorescent foci. B. Localization of endogenous R2 chromosomal reporter in filaments growing with low cipro. R2 loci localize as discrete CFP foci that may result of a smooth progression of the replication fork in regions downstream to the origin of replication. C. Visualization of chromosome morphology by DAPI staining at various concentrations of cipro (5, 20 and 100 ng/mL). When cipro was added at a concentration greater than 0.125x MIC, the chromosome dynamics was severely impaired. In all images, scale bar is 2 microns.

Figure S3: Isolation of offspring from the filamentous parent cells.
A. Fluorescent microscopy images of wild type gfp+ cells, grown on LB agar containing low cipro for 10 h (left image). The cells were scrapped off the plate using a sterile rake and resuspended in 5 ml LB added on the plate. The short offspring cells were separated from the filamentous parent cells by filtration using a 5 µm syringe filter (right image). Scale bar is 3 microns. Inset shows that 70% of progeny after filtration survives on low cipro (based on CFUs).

Figure S4: Tracking chromosome localization within filaments
Time lapse images of R2-CFP loci localization in E. coli cells, without cipro (A) and with low cipro (B-D). Time (in min.) is indicated. Scale bar is 1 micron (A-C) and 2 microns in in (D). Red circles surrounding R2 loci result from automated tracking analysis software (3). Dashed lines show the cell contours.

Supplementary Movie 1: E. coli (wt) bacteria growing in the presence of low cipro for 9 hours (544 min.). Microscopy images were taken using GFP channel every 2 min. The movie’s frame rate is 15 images per second. Scale bar is 10 microns

Supplementary Movie 2: E. coli (wt) bacteria growing in the presence of low cipro for 9 hours (544 min.). Microscopy images (Bright field) were taken every 2 min. The movie’s frame rate is 15 images per second. Scale bar is 10 microns
**Supplementary Movie 3:** Time course of chromosome (R2-CFP loci) localization in filamentous *E. coli* (wt) cells growing in the presence of low cipro. Images were taken every 5 min. The movie's frame rate is 3 images per second.

**Supplementary Table 1:** Bacterial strains used in this work

<table>
<thead>
<tr>
<th>Name</th>
<th>Genotype</th>
<th>Source, references</th>
</tr>
</thead>
<tbody>
<tr>
<td>JEK1036</td>
<td><em>E. coli</em> W3110 Plac-gfp</td>
<td>(4)</td>
</tr>
<tr>
<td>JEK1037</td>
<td><em>E. coli</em> W3110 Plac-mrfp1</td>
<td>(4)</td>
</tr>
<tr>
<td>QZ030</td>
<td><em>E. coli</em> W3110 Plac-gfp containing 4 SNPS in gyrA marR, and rbsA genes</td>
<td>(5)</td>
</tr>
<tr>
<td>SMR14510</td>
<td><em>E. coli</em> W3110 Plac-mrfp1 ∆upp::PsulA-gfp cat</td>
<td>This work</td>
</tr>
<tr>
<td>SMR17060</td>
<td><em>E. coli</em> W3110 malB::Tn9 Plac-gfp lexA3</td>
<td>This work</td>
</tr>
<tr>
<td>RHA050</td>
<td><em>E. coli</em> W3110 Plac-gfp marRΔ1</td>
<td>This work</td>
</tr>
<tr>
<td>RHA040</td>
<td><em>E. coli</em> W3110 Plac-gfp marRΔ11</td>
<td>This work</td>
</tr>
<tr>
<td>RHA020</td>
<td><em>E. coli</em> W3110 Plac-gfp gyrAA84P</td>
<td>This work</td>
</tr>
<tr>
<td>ZW</td>
<td><em>E. coli</em> AB1157 lacO240-Hm + tetO240-Gm array (R2), PdnaA lac-mCherry, Plac tetR-mCerulean</td>
<td>Gift from Dr. Sherratt's lab (1,2)</td>
</tr>
</tbody>
</table>

Strain SMR14510 was constructed by P1 transduction of ∆upp::PsulA-gfp cat from strain SMR10762 into JEK1037 and confirmed by PCR. Strain SMR10762 was constructed by first using short homology recombineering (6) to insert the chloramphenicol acetyltransferase (cat) gene from pKD3 (6) into pJP30 from SMR10697 with primers 5’ GTT GAA GGC ATG CAA GGA GAT GGC GCC CAA CTG TGT AGC TGG AGC TGC TTC G and 5’ TGA GCG CTT GTT TCG GCG TG TGG GTA TGG TGG CCA TAT GAA TAT CCT CCT TA, creating pJP30 cat. From this plasmid, PsulA-gfp cat was amplified with primers 5’ TAC CAA AGA TTT TGT CAC CGG CAT CGC CGA GCC CGC GAA GTC TAG ACT GTA ACA GCA GCT GGG ACT GGA GTC TAG GAT GGG CCC GAA TGT GTA AAG TCA AAC ACC ACT CTG CAA ACA GCT GGG ACT GGA GTG CTA GAT CCT GCA G and inserted into the genome replacing base pairs 51-580 of the upp gene of strain SMR4562 by Red-mediated short- homology recombination. Constructs were confirmed by PCR and flow cytometry.

**Supplementary Table 2:** Primers used for sequencing

<table>
<thead>
<tr>
<th>gene</th>
<th>Sequenced product size</th>
<th>Primer name</th>
<th>Primer Sequence 5’-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>gyrA</td>
<td>641 (-6; +635)</td>
<td>gyrA_FOR</td>
<td>GGTTAGATGAGCGACCTTGC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>gyrA_REV</td>
<td>ATGTGTCCATCAGCCCTTC</td>
</tr>
<tr>
<td>marR</td>
<td>559 (-45; +514)</td>
<td>marR_FOR</td>
<td>GGCACAATATTCCCTGCAA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>marR_REV</td>
<td>GTCCCTGATCCAGTCCAAG</td>
</tr>
<tr>
<td>acrR</td>
<td>732 (-21; +711)</td>
<td>acrR_FOR</td>
<td>CGGCTGGAAATTCACTGAA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>acrR_REV</td>
<td>CGCAGTGAAACCAGAATAGCA</td>
</tr>
<tr>
<td>rbsA</td>
<td>182 (+1325;1507)</td>
<td>rbsA_FOR</td>
<td>AAGCCGATGCGCTGAG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rbsA_REV</td>
<td>TTACTCTGATTCACGC</td>
</tr>
</tbody>
</table>
**Supplementary Methods:**

**Bacterial strains and growth**

Cipro was solubilized in 0.2N HCl solution and diluted in sterile water to proper concentrations. 0.5 mM IPTG was used to induce the expression of GPF. P1 phage transduction was carried out as described (7). To isolate offspring cells from filamentous cells, overnight bacterial cultures were subcultured 1:100 in LB and grown until they reached mid-exponential phase. 100 microliters of each culture were plated on a LB agar plate containing low cipro. Plates were incubated for 24h at 37°C. Bacteria were scraped off LB agar plates, carefully resuspended in 5 mL of fresh LB medium (containing no cipro) and filtered out by using a 5 µm sterile syringe filter (Sterlitech). The filtered cell culture was imaged under the microscope to verify that it contains no filaments (Fig. S4).

**Imaging**

From long time-lapse movies we measured the position of the septation site along the filaments, the length of the filament at which the first budding event occurred as well as the number of budding events and the time at which they occur per filament, by using Image J analysis software. Filament death was visualized using 1µg/mL PI added to the cipro-containing agarose pad. Images were taken at times 0, 5h and 24h. For nucleoid visualization, cells were grown in liquid LB medium in the presence of low cipro for 4h at 37°C (no shaking). Measurements of DAPI intensities of completely segregated chromosomes were obtained by using NIS-elements image analysis software.

For fluorescent R2 loci tracking, automatic tracking of the fluorescent signal was performed using a custom MatLab code adapted from the U-track software of Jaqaman et al. (3). The users performed identification of the cell centers interactively because automatic recognition in bright field images could not be reliably achieved. Output from the program consists of a matrix of coordinates for the loci position.

**Sequencing**

Colonies were streaked out for isolation and grown for 24h at 37°C. Colony PCR was then performed on the resistant clones to get DNA templates of genes rbsA (encoding a ribose ABC transporter), marR (encoding a repressor of AcrA/B-TolC efflux pump expression), acrR (encoding a repressor of AcrA/B-TolC efflux pump expression), gyrA (encoding topoisomerase Gyrase A) and sulA (encoding a division inhibitor) with the following primers rbsAFOR, rbsAREV, marRFOR, marRREV, acrRFOR, acrRREV, gyrAFOR and gyrAREV, PsulAFOR, sulAREV, respectively. Primer sequences are listed in supplementary Table 2 (Table S2). For the acrR, marR, rbsA and sulA genes, the entire coding region was sequenced. For gyrA, the fragment that contains the Quinolone Resistance Determining Region (QRDR), the most consistent location of ciprofloxacin resistance mutations in gram-negative bacteria was sequenced. The PCR products were purified using the Qiaquick gel extraction kit (Qiagen) and sent to sequencing with the respective sequencing primer rbsAFOR, marRFOR, acrRFOR or gyrAFOR (Table S2). Deletions in marR coding region were found at nucleotide 377 (marRΔ1) and from nucleotide 302 to 312 (marRΔ11). Mutation in gyrA was found in the QRDR at nucleotide 248.
References:

Figure S2

A) Diagram of a typical bacterial cell with labeled regions: oriC, ter, R2, lacZ.

B) Overlay image of bacterial cells with labeled regions: overlay and R2-CFP.

C) Fluorescence images showing DAPI, GFP, with ciprofloxacin concentrations: cipro 5, cipro 20, cipro 100.
Figure S3

R2-CFP localization vs time (min.)

No cipro

Low cipro
Figure S4

A

WT cells (gfp+): Before filtering  WT cells (gfp+): After filtering

scale bar 3um